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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Barbara Ensoli	Confirmation No.:	9400
Application No.:	09/555,534	Art Unit:	1648
Filed:	May 31, 2000	Examiner:	Humphrey, Louise Wang Zhiying
For:	HIV TAT, OR DERIVATIVES THEREOF FOR PROPHYLACTIC AND THERAPEUTIC VACCINATION	Attorney Docket No.:	11340-003-999

DECLARATION OF MAURO MAGNANI, Ph.D.
UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, MAURO MAGNANI, Ph.D., do declare as follows:

1. I am currently Professor of Biochemistry, Director of Centre of Biotechnology, Vice-Rector of the University of Urbino, Urbino, Italy. I have over thirty years of experience as a biochemist in the research and development of products and applications useful in the biotechnology and pharmaceutical industries. I am included in the official list of professional biologists in Italy with n. 017484 "Ordine Nazionale Biologi," and I am a Technical Director nominated by the "Agenzia Italiana del Farmaco, AIFA" with n. AIDT-19/2005. My education and experience are summarized on my Curriculum Vitae, which is attached hereto as Exhibit 1.

2. I have collaborated with Dr. Barbara Ensoli, who is the inventor of the above-identified application No. 09/555,534 (hereinafter "the '534 application"), in the development of methods to produce recombinant, biologically active Tat protein. I also supervise and have supervised the production of such recombinant, biologically active Tat protein, according to good manufacturing practices (GMP), for use in human clinical trials.

3. I have reviewed the specification of the '534 application and have been asked to evaluate whether one skilled in the art as of December 1, 1997 could obtain a composition containing a biologically active Tat that is pharmaceutically acceptable for administration to a human, based on the teaching of the specification and knowledge common in the art as of December 1, 1997, and using only routine experimentation. As discussed in detail and for the reasons set forth below, and based on certain assumptions set forth below, it is my judgment and opinion that one skilled in the art as of December 1, 1997 could obtain a composition containing a biologically active Tat that is pharmaceutically acceptable for administration to a human, based on the teaching of the specification and knowledge common in the art as of December 1, 1997, and using only routine experimentation.

4. The specification of the '534 application teaches purification of biologically active recombinant Tat produced in *E. coli* (see, *e.g.*, page 25, line 14; and page 27, line 4). The specification of the '534 application at page 25, lines 5-25, describes two methods for purification of biologically active Tat: (1) a first method based on successive steps of ion-exchange chromatography and high-pressure liquid chromatography (HPLC); and (2) a second method based on heparin affinity chromatography, with the second method noted as preferable (page 26, lines 4-8).

5. For purposes of this Declaration, I have assumed that a Tat composition obtained from use of the first method will contain acetonitrile and trifloroacetic acid (TFA) as process impurities, and that a Tat composition obtained from use of the second method will contain phenylmethylsulfonyl fluoride (PMSF). I am informed that the presence of acetonitrile/TFA and PMSF, respectively, would render the Tat compositions obtained from the first and second methods not pharmaceutically acceptable for administration to a human.

6. As discussed in Paragraph 4 above, the specification of the '534 application at page 25, lines 5-26, describes three chromatographic procedures which can be used to purify a biologically active Tat protein: ion-exchange chromatography, HPLC, and heparin affinity chromatography. It was commonly known in the art as of December 1, 1997 that a combination of purification steps should yield improved purification over a single one of the purification steps. It was also commonly known in the art as of December 1, 1997 that, when isolating a recombinant protein from bacterial cells, improved purification would, for example, decrease levels of endotoxin in the resulting protein preparation, a result known to

be desirable when purifying a protein for human therapeutic use. Low levels of endotoxin are, in fact, required by regulations for human use.

7. A person skilled in the art, when reading the specification of the '534 application, and thus choosing a combination of procedures to use for purification of biologically active Tat for human therapeutic use, would clearly avoid HPLC rather than heparin affinity chromatography, due to the known unsuitability of the commonly used acetonitrile/TFA solvent system in HPLC, since such person would expect that the use of PMSF in heparin affinity chromatography could be avoided much more easily than the use of the standard solvent system of acetonitrile/TFA in HPLC. Among those chromatographic procedures taught in the specification, the skilled person thus would be left with ion-exchange chromatography and heparin affinity chromatography.

8. In combining ion-exchange chromatography plus heparin affinity chromatography, a person skilled in the art also would know to perform the ion-exchange chromatography step before the heparin affinity chromatography step, since the commonly known general strategy in developing combination procedures for protein purification is to proceed from high-capacity procedures (*e.g.*, ion-exchange chromatography) to low-capacity procedures (*e.g.*, heparin affinity chromatography), as taught for example in Linn S., "Strategies and considerations for protein purifications," Methods Enzymol. 1990;182:9-15, under section entitled "Refined Procedures," at pages 14 and 15 (a copy of which is attached hereto as Exhibit 2).

9. As discussed in Paragraph 5 above, a Tat composition obtained from heparin affinity chromatography will contain PMSF, the presence of which would render said Tat composition not pharmaceutically acceptable for administration to a human. PMSF is a serine protease inhibitor used to protect proteins during purification from bacteria. Specifically, PMSF inhibits degradation of the purified protein by serine proteases which are released from the cell along with the protein during purification. A person skilled in the art would know that methods to reduce protease activity, commonly known in the art, could be employed, in order to avoid the use of PMSF. For example, the protein of interest could be expressed in a bacterial system that is deficient in proteases. See, *e.g.*, McGrath *et al.*, "Production of crystallizable human chymase from a *Bacillus subtilis* system," FEBS Lett. 1997 Aug 25;413(3):486-8 ("McGrath *et al.*"), a copy of which is attached hereto as Exhibit

3. McGrath *et al.* describes the use of a *Bacillus subtilis* strain deficient in seven extracellular proteases to produce human mast cell chymase (see Abstract).

10. The initial step(s) in purification also can be carried out at 4°C to reduce protease activity prior to separation from proteases in the cell lysate, *e.g.*, as described in James *et al.*, "Purification and biochemical characterization of a vacuolar serine endopeptidase induced by glucose starvation in maize roots," Biochem J. 1996 Nov 15;320 (Pt 1):283-92 ("James *et al.*"), under section entitled "Purification of the root-starvation-induced protease (RSIP)," at page 284, col. 1, line 1 (a copy of which is attached hereto as Exhibit 4); and Goldberg *et al.*, "Proteases in *Escherichia coli*," Methods Enzymol. 1981;80 Pt C:680-702 ("Goldberg *et al.*"), at page 687, last paragraph, lines 1-3 (a copy of which is attached hereto as Exhibit 5), or by "taking care that the temperature does not rise above 10°C" as described in Pirrotta *et al.*, "General purification schemes for restriction endonucleases," Methods Enzymol. 1980;65(1):89-95, at page 90, first paragraph, lines 3-5 (a copy of which is attached hereto as Exhibit 6). The authors of James *et al.* and Goldberg *et al.* each studied the effect of PMSF on the activity of their purified protease (see James *et al.*, page 288, col. 1, last paragraph; and Goldberg *et al.*, page 691, second paragraph under section entitled "Properties of Re," and page 695, under section entitled "Properties of So"). For this reason, they did not wish to include PMSF in the extraction buffer, and thus they performed procedures at 4°C to minimize the degradation of the protease.

11. Thus, a person skilled in the art as of December 1, 1997, based on the teaching of the specification and knowledge common in the art as of December 1, 1997, and using only routine experimentation, would know that (i) the ion-exchange chromatography and heparin affinity chromatography steps, as described in the above-identified application, could be combined to achieve improved purification over either alone, thus advantageously avoiding HPLC which involved solvents (*e.g.*, acetonitrile and TFA), the presence of which would render a Tat composition not pharmaceutically acceptable for administration to a human; and (ii) the initial steps of the purification procedures could be performed at a low temperature (*e.g.*, 4°C) and/or the proteins could be expressed in protein-deficient bacterial strains, to avoid proteolytic degradation of the Tat, thus advantageously avoiding the use of PMSF, the presence of which would render a Tat composition not pharmaceutically acceptable for administration to a human; to obtain a biologically active Tat that is pharmaceutically acceptable for administration to a human.

12. Therefore, in my judgment and opinion, a person skilled in the art as of December 1, 1997, based on the teaching of the specification and knowledge common in the art as of December 1, 1997, and using only routine experimentation, could obtain a Tat composition that is pharmaceutically acceptable for administration to a human.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application, and any patent issuing thereon.

Date: April 27, 2007

Mauro Magnani, Ph.D.

Curriculum Vitae – Prof. Mauro Magnani

MAGNANI Prof. Mauro, Ph.D. Italian, Professor of Biochemistry
BORN: April 9, 1953, Italy
LANGUAGES: Italian, English
EDUCATION: Univ. Urbino, Italy, Ph.D., 1976
PRIMARY POSITION: Professor of Biochemistry and Director Centre of Biotechnology.
PROFESSIONAL CAREER: Visiting Researcher, Dept. Biochemistry, Univ. Birmingham, 1980; Visiting Prof. Dept. Biolgy, Haifa, Israel, 1983; Asst. Prof. Univ. Urbino, 1977-82, Assoc. Prof., 1982-1986, Prof. 1986 - ; Dean, Faculty of Sciences University of Urbino 1995-2001; Director Interuniversity Consortium for Biotechnology (CIB) 1998-2004; Vice Rector of the University of Urbino 2001- .Include in the official list of professional biologist in Italy with n. 017484 "Ordine Nazionale Biologi". Technical Director nominated by the "Agenzia Italiana del Farmaco, AIFA" with n. AIDT-19/2005.
CURRENT RESEARCH: Development of new drug delivery and drug targeting systems; Protein turnover ubiquitination and regulation of gene expression; Mechanisms of drug resistance and drug toxicity; Modulation of NF-kB and gene expression by oligonucleotide decoys, vaccine development; nanobiotechnology in drug delivery.
PUBLICATIONS: over 300 articles published in international refereed scientific journals;
Co-editor of three books:
"*Red Blood Cell Aging*", Plenum Press, N.Y., 1991, pp. 383.
"*The Use of Resealed Erythrocytes as Carriers and Bioreactors*", Plenum Press, N.Y., 1992, pp. 361.
"*Erythrocyte Engineering for Drug Delivery and Targeting*", Landes Bioscience, 2002.
REFEREE: Programmes of the E.U.; The International Science Foundation (U.S.A.); Target Project "Biotechnology" of the National Research Council (C.N.R.).; Member of the Project "Patologia clinica e terapia dell'infezione da HIV" of the Italian Ministry of Health; PRIN and FIRB Projects of Italian Ministry of University and Research; Member of Committee Post Genoma (C.N.R); Include in the "Albo degli Esperti" of M.I.U.R. and Eureka Projects of EU.
REVIEWER: Biotechnology and Applied Biochemistry; Nature Biotechnology; Drugs; Leukemia; European Journal Haematology; Biochimica et Biophysica Acta; Blood; Journal of Cellular Engineering; Journal of Internal Medicine; Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology; Mechanisms of Ageing and Development; Antiviral Research; Journal of Chromatography; Journal of Biological Regulators and Homeostatic Agents; Life Sciences; Biochemistry; International Journal of Biochemistry and Cell Biology; Human Gene Therapy; European Journal of Biochemistry; Clinical Pharmacokinetics; Autoimmunity; Oncogene; Haematologica; J. Controlled Release; Editorial Board: Current Drug Targets, Biotechnology.

PATENTS

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US Patent 5,753,221

M. Magnani, L. Rossi "*Transformed erythrocytes, process for preparing the same, and their use in pharmaceutical compositions*"

US Patent N. 6.139.836
Mauro Magnani, Ivo Panzani, Leonardo Bigi, Andrea Zanella "*Method of encapsulating biologically active agents within erythrocytes, and apparatus therefor*".
Assignee: Dideco S.p.A., Mirandola, Italy

European Patent N. EP98830479.6
M. Magnani, G. Brandi, A. Fraternale, A. Casabianca "*Pharmaceutical composition or composition package containing a pyrimidine nucleoside analogue and a purine nucleoside analogue*".

Brevetto C.N.R. N. RM92 A 000377
M. Magnani "*Antigeni legati alla superficie esterna di eritrociti e procedimento per la loro preparazione*"

Brevetto C.N.R. N. RM 93 A 000474
M. Magnani "*Eritrociti incorporanti alcool ossidasi e loro uso nelle intossicazioni da metanolo*"

Brevetto C.N.R.
M. Magnani, L. Rossi, G. Brandi, E. Millo, G. Damonte, U. Benatti, A. De Flora "*Profarmaco di acyclovir e suo uso in composizioni farmaceutiche*"

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M. Magnani, C. Fiorucci, P. Filippone, G. Brandi, M. Paiardini. "*Derivato tetramerico dell'indol-3 carbinolo ad attività anticancerogena e metodo di sintesi del derivato stesso*".

Brevetto di Invenzione N. TO2001A01077 – 16/11/2001
M. Magnani, F. Graziano, A. Ruzzo "*Mutazioni della linea germinale nel promotore del gene della E-caderina e metodi di diagnosi per individuare una maggiore suscettibilità al carcinoma gastrico*".

Brevetto N. TO2003A001048 – 30/12/2003 - PCT/EP/2004/053726 – 29/12/2004
U.Benatti, G. Brandi, E. Garaci, M. Magnani, E. Millo, A.T. Palamara, L. Rossi. "*Derivati del glutathione e loro utilizzo per il trattamento di malattie virali*".

[2] Strategies and Considerations for Protein Purifications

By STUART LINN

The budding enzymologist is generally surprised by the time necessary to develop a protein purification procedure relative to the time required to accumulate information once the purified protein is available. While there is no magic formula for designing a protein purification, some forethought can help to expedite the tedious job of developing the purification scheme. This chapter is designed to point out some considerations to be undertaken prior to stepping up to the bench. Once at the bench, the subsequent chapters of this book as well as two other recent publications concerning enzyme purification[1,2] should serve as a guide.

Preliminary Considerations

What Is the Protein To Be Used For

In these days of the biotechnology revolution, the required amount of purified protein may vary from a few micrograms needed for a cloning endeavor to several kilograms required for an industrial or pharmaceutical application. Therefore, a very major consideration is the amount of material required. One should be aware of the scale-up ultimately expected, and the final scheme should be appropriate for expansion to those levels. There are very real limitations to how far a procedure can be scaled up. These limitations are brought about not only by considerations of cost and availability of facilities, but also by physical constraints of such factors as chromatographic resin support capabilities and electrophoresis heating factors. As outlined below, individual steps of the procedure should flow from high-capacity/low-cost techniques toward low-capacity/high-cost ones. Nonetheless, in some cases two procedures may be required: for example, one to obtain microgram quantities for cloning and a second to produce kilogram amounts of the cloned material. The protein chemist should remain flexible for adopting new procedures when such changes are warranted.

Another consideration is whether the protein must be active (an enzyme, a regulatory protein, or an antibody, for example), whether it must

[1] R. K. Scopes, "Protein Purification, Principles and Practice," 2nd Ed. Springer-Verlag, New York, 1987.

[2] R. Burges, ed., "Protein Purification, Micro to Macro." Alan R. Liss, New York, 1987.

be in a native configuration, but not associated with an activity, or whether it need not be in any specific configuration (a small peptide or a peptide to be utilized only for obtaining sequence information, for example). The techniques employed should be as gentle as is necessary, but, whenever possible, some of the harsher but often spectacularly successful procedures such as those which involve extremes of pH, organic solvents, detergents, or hydrophobic or strong affinity chromatographic media should also be used.

Assays

Possibly the most important preliminary step is to develop appropriate assays. The success of the purification is often most dependent on this. Five considerations come to mind: sensitivity, accuracy, precision, substrate availability, and cost.

Sensitivity is often the limiting factor as the protein becomes diluted into column effluents, etc. Before beginning a step, the likely dilution and losses ought to be estimated and the ability to detect the protein after a reasonably successful procedure ought to be possible.

Accuracy and precision are often compromised in these days of fast technology, but clearly these items must be controlled to the extent that the assay is reliable for assuring recovery of material and reproducibility.

Specificity is usually a problem early in the purification. Often, however, substrates can be simplified or controls omitted as the purification progresses.

Substrate availability and cost refer to the practicality of the assay: Can enough substrate be prepared to perform the entire purification without interruption? Stopping to prepare more substrate or skimping on material usually results in disaster. On the other hand, assays at certain steps in the purification might be modifiable, e.g., leaving out specificity controls at later stages or assaying alternate chromatography fractions.

There is a recent trend not to use assays for protein activity, but to purify a gel band or an antigen instead. Although this tactic might be appropriate in instances where activity is not being sought, it is to be strongly discouraged when activity is in fact what is desired. It cannot be emphasized strongly enough that an activity assay is necessary to obtain optimal yields of activity, be it one associated with an enzyme, a DNA-binding protein, an antibody, or a hormone.

A final comment pertains to the protein assay. Again, the goals are simplicity, reproducibility, specificity, and reliability. Accuracy is generally compromised, as no commonly used assay is absolute with regard to all proteins. With crude fractions, color reactions are probably best.

TABLE I
ADDITIONS TO PROTEIN SOLVENTS

Class	Examples	Purpose
Buffer		Stability
Salt	KCl, NaCl, $(NH_4)_2SO_4$	Stability
Detergents	Deoxycholate	Stability, solubility
Surfactants	Triton X-100	Stability
Glycerol, sucrose		Stability; allows storage below 0° in liquid state
Sodium azide		Bacteriostatic
Metal chelators	EDTA (ethylenediaminetetraacetic acid), EGTA [ethylene glycol bis(β-aminoethylether) *N,N'*-tetraacetic acid]	Stability
Sulfhydryl agents	2-Mercaptoethanol, dithiothreitol	Stability
Ligands	Mg^{2+}, ATP, phosphate	Stability
Protease inhibitors	PMSF (phenylmethylsulfonyl fluoride), TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone), TLCK (N^{α}-*p*-tosyl-L-lysine chloromethyl ketone)	Stability

While the Bradford method[3] is by far the simplest of these, in our laboratory we find it to be unreliable with crude fractions from animal cells or when detergents are present. For column effluents, ultraviolet absorption is optimal: it is simple, sensitive, and does not consume the material. For extremes of sensitivity, wavelengths between 210 and 230 nm can be utilized.[4,5] Again, protein assay procedures can and often must be changed as the purification progresses.

What Should Be Added to the Buffers

Once a purification scheme is developed, there is great resistance to modifying it, as modification requires laborious trial runs. The usual response to "why is the protein suspended in *x*?" is "if I leave it out, I don't know what will happen." The obvious lesson is to add something only with good reason in the first place.

Solutes are added usually to improve stability, prevent the growth of microorganisms, reduce the freezing point, or keep the protein in solution. Table I lists several classes and examples of such additions. It is well

[3] M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
[4] W. J. Waddell, *J. Lab. Clin. Med.* **48**, 311 (1956).
[5] M. P. Tombs, F. Souter, and N. F. MacLagan, *Biochem. J.* **73**, 167 (1959).

worth the effort to carry out stability studies (e.g., heat inactivation or storage trials) in order to learn how to maintain a stable protein. Two notes of caution: (1) optimal storage conditions change with purification; (2) optimal storage conditions need not relate to optimal conditions for activity. Indeed, additions which stabilize a protein often inhibit it when added to activity mixes. Of course, the latter situation must be considered when utilizing the protein—interfering substances will have to be removed or "diluted out" during utilization of the protein.

In our experience, reducing agents are particularly effective with bacterial enzymes which derive from a reducing environment, whereas mammalian cell enzymes take kindly to surfactants and protease inhibitors. Fungal proteins also respond to protease inhibitors. Optimal pH and salt concentrations vary. Most enzymes prefer the lowest temperature allowable: 0° (on ice, not in a refrigerator) or −20° with glycerol or sucrose present. If frozen, storage above liquid nitrogen or at −70° is often best. Special precautions which must be taken for purification and stabilization of large protein complexes are noted in Section IX of this volume.

A final note concerns the containers used for purified proteins or purification fractions. Glass should not be used with very dilute solutions, plastic tubes being better. In our experience, polypropylene-based plastics are superior to polyethylene ones, and polystyrene or other clear plastics are less satisfactory. Be sure to have tight-fitting caps if storage is in "frost-free" freezers.

Contaminating Activities

Often proteins need not or cannot be obtained in a pure state, but particular interfering activities (e.g., nucleases, protease, phosphatases) must not be present. In our experience, attempting to purify one activity against one or more others by doing multiple types of activity assays as a criterion of purity is an extremely frustrating endeavor. Instead, purifying so as to optimize yield and specific activity (units/mg protein) with selective choice of fractions only at the last or at most penultimate step is more likely to be satisfactory.

Source of Protein

When the source of a protein is not absolutely dictated, careful consideration of the source is worth the time and effort, and trial extracts from a number of sources should be done.

The cost and availability of the source, particularly if a largely scaled up preparation might be desirable in the future, should also be considered

as well as the genetic knowledge and technology available for the organism should regulatory and/or gene sequence manipulations be envisioned. If the protein is to be overexpressed, is a bacterial or fungal cell better? Which one? What special precautions are necessary for each organism? Will the protein be appropriately processed?

Once at the bench, several sources should be tested for total yield of activity (per gram of starting material or per unit cost), the starting specific activity (units/mg protein), and the stability of the protein. In the extreme case, the classical microbiological approach of isolating microorganisms with unique growth requirements might lead to unexpected success.

Preparing Extracts

Preparing extracts is discussed in Section IV of this volume so only general considerations will be noted here. In our experience, the manner in which cells are disrupted has a profound and unpredictable effect on the yield and quality of the protein preparation. Trials are clearly necessary.

Thought should always be given toward scaling up the preparation, and how the disruption procedure will or will not adapt to being scaled up. Will the volumes or time required become unreasonable? Can a subsequent clarification step also be conveniently scaled up?

In general, volumes should be kept as small as possible, i.e., extracts as concentrated as possible. Tissue, cell type, or organelle fractionation is almost always worthwhile prior to disruption. Finally, consideration should also be given to the substance in which the starting material is suspended so that the protein desired is soluble and/or stable. Of course, the contents of the suspension buffer should not interfere with the subsequent step(s) in the purification procedure.

Bulk or Batch Procedures

These procedures are almost always utilized early in the purification as they are often most effective in removing nonprotein material and are most amenable to the large volume and amounts of material that exist in earlier stages of the preparation. A great deal of effort went into designing these steps in the early days of protein chemistry, and much frustration can probably be avoided by reinstituting some of these old-fashioned procedures.

Section VI of this volume outlines some of these approaches. Drastic methods such as heat, extremes of pH, or phase partition with organic

solvents might be particularly effective with stable proteins, though subtle forms of damage may be difficult to foresee or to detect. Gentler procedures include phase partition with organic polymers, "salting out," or addition of ion-exchange resin as a slurry. Batch elution from large, high-capacity ion-exchange columns might also be effective. The time expended in developing and optimizing these early steps is always worthwhile—even a factor of two increase in specific activity may decide the feasibility of a subsequent step from both cost and technical considerations.

Refined Procedures

Once the bulk methods have yielded a protein preparation which is reasonably free of nucleic acids, polysaccharides, and lipids, the preparation becomes amenable to the more interesting and spectacular procedures which have been developed in recent years. The general strategy is to proceed from high- to low-capacity procedures and to attempt to exploit specific affinity materials whenever possible.

Applications and technical details for these procedures are noted in Sections VII, VIII, IX, and XI of this volume, and will not be described here beyond citing examples. As a general consideration, in proceeding from one procedure to the next, one ought to reduce as much as possible the necessity for dialysis and concentration. Hence, procedures that separate by size can also be exploited to remove salt. Procedures utilizing high-capacity resins can concentrate proteins as well as purify them, or resins from which proteins elute at low-salt concentrations can be directly followed with resins to which the protein binds at higher salt concentrations. Also, some steps (e.g., sedimentation through gradients of sucrose or glycerol) may leave the protein in a medium which might be ideal for long-term storage, but difficult (or appropriate) for utilization in a subsequent step. Finally, interchanging the order of the steps of a procedure can, and often does, have a profound effect on the success of a purification scheme.

Some procedures which cannot be effectively scaled up [e.g., sedimentation, or high-performance liquid chromatography (HPLC)] can be carried out with small aliquots of the preparation, but only if left to the final stages. (In some instances the utilization of aliquots is desirable, the less purified fractions may be more stable to long-term storage.)

High-Capacity Steps

Generally, these include ion-exchange resins or very general affinity agents such as dyes or glass. When used for large amounts of material,

ion-exchange resins can often be successfully reutilized at a later stage for additional purification (especially if the pH is changed) or for concentration.

Intermediate-Capacity Steps

These might include the hydrophobic resins for which long chromatographic times reduce activity yields. Many affinity agents (bulk DNA or simple DNA sequences, immunoaffinity, or ligands of a protein) fall into this class. In these instances, thought and effort must be given to finding materials that can successfully elute the protein without destabilizing or inactivating it. Gel filtration should also be considered as a step with intermediate capacity.

Low-Capacity Steps

Affinity steps utilizing valuable ligands such as substrate analogs, complex DNA sequences, and lectins might be included here. Also included are isoelectric focusing (precipitation is often a problem with moderate amounts of protein), electrophoresis, HPLC (which in our hands is difficult to scale up without loss of resolution), and ultracentrifugation. Very small hydrophobic columns might also be successful where larger ones have failed.

Conclusions

Though protein purification is often a difficult and frustrating process, its rewards are great. Moreover, with the continual development of new technology, the commercial availability of materials utilized for purification procedures, and the availability of genetically altered sources of material, the future bodes well for simpler procedures accompanied by greater rewards and indeed for protein chemistry as well.

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Production of crystallizable human chymase from a *Bacillus subtilis* system

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Received 17 June 1997

Abstract A *Bacillus subtilis* strain deficient in seven extracellular proteases was used to produce human mast cell chymase and is a viable expression system for serine proteases and other classes of proteins. Chymase is produced at 0.3–0.5 mg/l and is purified by three chromatography steps. Two crystal forms of PMSF-treated chymase were optimized. The first is C2 with $a = 47.94$ Å, $b = 85.23$ Å, $c = 174.18$ Å, $\beta = 96.74°$, and diffracts to at least 2.1 Å, while the second is P212121, with cell dimensions $a = 43.93$ Å, $b = 58.16$ Å, and $c = 86.09$ Å, and a diffraction limit of approximately 1.9 Å. The first crystal form has either three or four molecules/asymmetric unit, while the second has one molecule/asymmetric unit.

Key words: Protease; Expression; Angiotensin; Mast cell; X-ray diffraction

1. Introduction

Human chymase is a mast cell serine protease with chymotrypsin-like specificity [1,2]. The enzyme is initially synthesized as an inactive precursor but is ultimately stored in, and released from, the secretory granules of mast cells as a mature enzyme [3–5]. Processing of the precursor appears to involve removal of a signal peptide followed by DPP1-mediated removal of the Gly–Glu propeptide in a heparin-dependent reaction [6,7]. Human chymase has been implicated in a variety of physiological and pathological processes including vasoactive peptide processing [8,9], tissue remodeling [10], activation of proIL-1β [11], and release of latent growth factors from the extracellular matrix [12]. While the parent chymotrypsin shows a relaxed substrate specificity, with clear preferences observed only for the P1 substrate position, chymase is a much more selective enzyme. Specificity for Phe, Tyr, or Trp at P1 remains, but is then limited by strict requirements for the extended substrate-binding site. For example, human chymase activates angiotensin I to angiotensin II by cleavage of a Phe–His bond, but does not degrade angiotensin II by clipping a Tyr–Ile bond, as do chymotrypsin, and chymases from other species [9]. To date, only one *chymase* gene has been identified in humans [13], although a Cys-to-Ser variation at position seven of the mature enzyme has been reported [14]. This is the first report of crystallization of human chymase. An atomic resolution structure of human mast cell chymase will provide useful information for understanding this enzyme's substrate specificity requirements and may shed light on the details of activation by DPP1, and the stabilizing role of heparin within the mast cell granule.

Production of milligram quantities of pure chymase was a prerequisite for the structural studies. Although a variety of expression systems have proven useful for serine proteases, many are unsuitable for one reason or another. For example, *Escherichia coli* can produce serine proteases to high yield, but many are then found inextricably complexed with the periplasmic inhibitor, ecotin [15,16]. Other reported expression systems for human chymase involve substantial downstream processing to recover limited amounts of active enzyme, e.g. refolding and proteolytic processing of an insoluble fusion protein [17] or proteolytic activation of secreted prochymase with cell extracts [6] or DPPI [7]. The *B. subtilis* system described herein yields mature chymase, ideally suited for crystallization because of the lack of glycosylation. *B. subtilis* has been successful at producing other serine proteases (B.F.S., unpublished results), and is a heretofore untapped resource as an expression system.

2. Methods and results

2.1. Expression

E. coli XL-2 Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^r) Amy Cam^r]} (Stratagene) or MM294 (*endA1 thi-1 hsdR17 supE44*) [18] were used as hosts for the vector constructions. Bacteria were transformed and plasmid DNA was purified as described elsewhere (B.F.S., manuscript in preparation).

B. subtilis WB751 (*trp* Δ*isp-1* Δ*nprA* Δ*aprE* Δ*epr* Δ*bpf* Δ*mpr*-Cam^r Δ*nprB-Ery*^{r} Δ*vpr*), a strain deficient in seven extracellular proteases and one intracellular protease, was used for the production of human mast cell chymase C7S. This strain was constructed by deleting the *vpr* gene [19] from *B. subtilis* WB600 (*trp* Δ*isp-1* Δ*nprA* Δ*aprE* - Δ*epr* Δ*bpf* Δ*mpr*-Cam^r Δ*nprB*-Ery^r) [20]. To accomplish this, two ≈1.2 kb PCR fragments, corresponding to either a 5′- or 3′-portion of the *vpr* gene, were generated by Taq polymerase (Perkin-Elmer) with the primers GATATCCAGAATTCCACAAACTCTATCTA-TATTTTGTATACGAA and GCCGTTCGGATCCTCAATCACT-TATGAGGTAACAGCGACAACGCCTTCTGACATGGC, or the primers GATACGTGGATCCTTAAGCCTGATATTTCCGCGCC-AG and GTATCCCTGCAGGAGTCCTCGTATGAAGCTGTTCG-G (ABI 394 DNA/RNA Synthesizer, Perkin-Elmer), respectively, using chromosomal DNA from *B. subtilis* BG2036(Δ*nprA* Δ*aprE*) (a generous gift from Genencor International, Inc.) as a template. The two PCR generated fragments (*Eco*RI–*Bam*HI and *Bam*HI–*Pst*I) were inserted in pBluescript SK+ (Stratagene) with the *kanamycin* gene (≈1.4 kb *Eco*RI–*Sal*I fragment) from pJM114 [21]. The deletion of amino acids 272–451 of the Vpr protease and the introduction of stop codons in all three frames after amino acid 271 were verified by DNA sequencing (ABI 373A DNA Sequencer, Perkin-Elmer). The resultant vector was used to transform *B. subtilis* WB600 cells. Clones with the vector integrated into the chromosome were selected by plating on Luria agar plates with 10 μg/ml kanamycin. Integration at the *vpr* gene locus was determined by screening the chromosomal DNA of the transformants using PCR primers that flank the *vpr* gene. Positive

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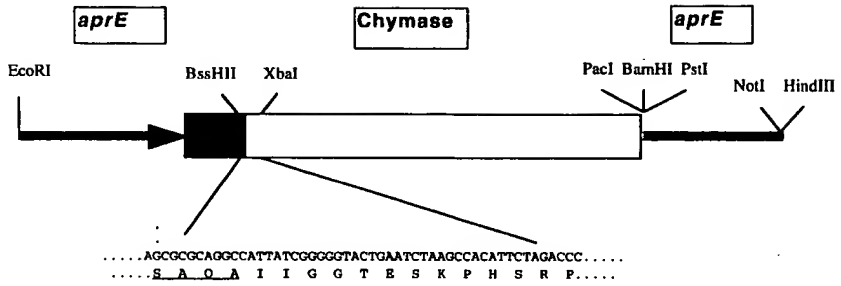

Fig. 1. A schematic representation of the expression cassette used to produce human mast cell chymase C7S in *B. subtilis*. Expression is driven by the *B. subtilis aprE* promoter [28] shown as the thick black arrow. The *chymase* gene is represented by the white box and downstream is the transcription terminator from the *B. amyloliquefaciens aprE* gene [29] shown as a thick black line. The DNA and protein sequence of the *aprE* signal sequence (underlined) fused to the mature *chymase* gene is shown below the schematic figure. Amino acids 9–30 of the signal sequence are derived from the *B. amyloliquefaciens aprE* gene.

clones were grown in Luria broth for 6–14 h then used to inoculate another 15 ml of fresh Luria medium (no kanamycin selection). The re-inoculation process was repeated for up to 2 weeks. Colonies that eventually lost the kanamycin resistance were screened by PCR in order to select clones where the native *vpr* gene was replaced with the deleted version.

A DNA fragment of the human *chymase* gene [5] was generated by PCR with the primers GGGAATATTATCGGGGGTACCGAATGCAAGCCACATTCTAGACCCTACA and GATCGTTAATTAATTTGCCTGCAGGATCTGGTTGATCCAGGG using a cDNA library constructed from ascites tissue as a template [22]. The ≈700 bp *Xba*I–*Pac*I gene fragment was ligated into the *Bss*HII and *Pac*I sites of the *B. subtilis* expression vector pBNppt (B.F.S., manuscript in preparation) using a *Bss*HII–*Xba*I synthetic DNA linker. This linker also substitutes the cysteine residue with serine at amino acid seven of the mature chymase since it was discovered that native human chymase with a free thiol group is not effectively produced in prokaryotic systems (J.M.C. and B.F.S., unpublished results). The entire sequence of the gene was verified by sequencing both DNA strands. A schematic representation of the expression–secretion construction is shown in Fig. 1.

2.2. Fermentation and purification

Chymase-producing *B. subtilis* was grown in 4 l of baffled shake flasks (Bellco) at 37°C, 270 r.p.m. A rich medium [23] was used for production and consisted of 3% (w/v) tryptone (Difco), 2% (w/v) yeast extract (Difco), 3% (w/v) glucose, 150 mM potassium phosphate, pH 7.4, 10.0 μg/ml neomycin. An inoculum (1 l) was grown overnight and

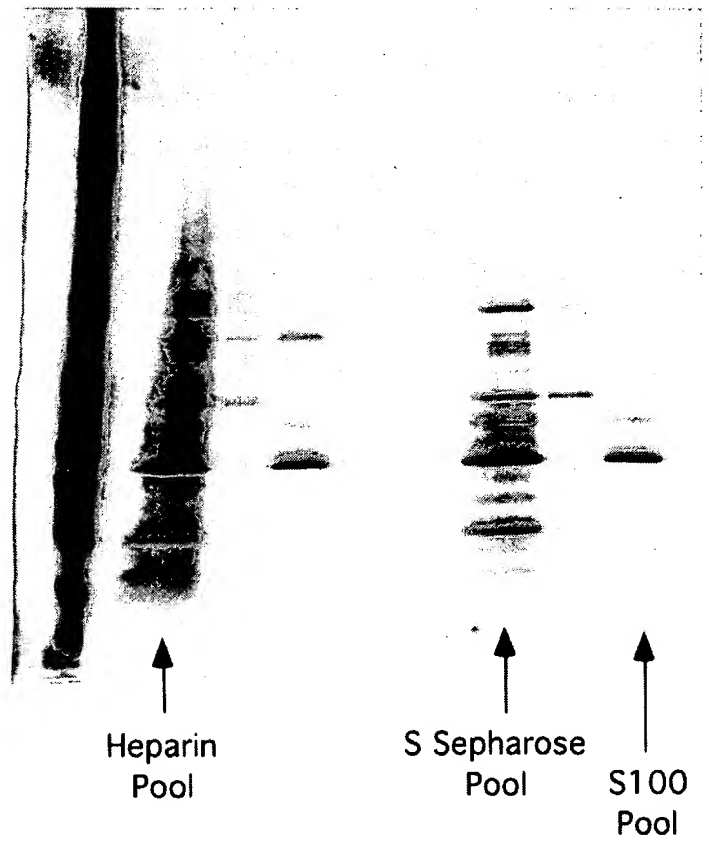

Fig. 2. A silver-stained 4–20% SDS-PAGE gel showing chymase at various purification steps. Chymase is the major protein band seen in the lane containing the pool from the S-100 chromatography step.

150 ml was used to seed each of six flasks containing 1 l of media. Cultures were grown for 6 h and harvested by centrifugation at 7200 r.c.f. The supernatant was filtered by passage through a 0.2 μm Sartobran-PH capsule filter (Sartorius, Edgewood, NY). Approximately 0.3–0.5 mg of purified chymase was produced per liter of fermentation broth, as determined by activity.

Chymase activity was measured by hydrolysis of suc-Val-Pro-Phe-SBzl (Bachem) with 4,4'-dithiodipyridine (Sigma), in assay buffer (30 mM Tris-HCl, 1.0 M NaCl, 0.05% (v/v) Tween 20, 10% (v/v) DMSO, pH 8.0). Beads of Heparin Sepharose CL-6B (Pharmacia, Piscataway, NJ) were added to the filtered harvest supernatant at a ratio of up to 0.5 l of harvest/ml resin and suspended with mild agitation for 1 h. The beads were collected on a 0.8 μm filter (Nalgene), resuspended in 20 mM MES, 150 mM NaCl, pH 5.5, and packed into a 5-cm-diameter column. Chymase was eluted from the column using a gradient of 0.52–2 M NaCl over five column volumes in 20 mM MES, pH 5.5. Fractions which tested positive for chymase activity were pooled, and diluted to a conductivity of 25 mS/cm with 20 mM MES, pH 5.5. The diluted chymase pool was applied to a SP-Sepharose FF column (1.6×10 cm, Pharmacia) equilibrated in 20 mM MES, 0.4 M NaCl, pH 5.5. Chymase was eluted from the column using a gradient of 0.4–1 M NaCl over 10 column volumes in 20 mM MES, pH 5.5. Chymase-rich fractions eluted in a peak centered around 0.7 M NaCl, and were assessed for purity by SDS-PAGE and pooled. The SP-Sepharose FF chymase pool was applied to a Sephacryl S-100 HR (2.6×92 cm, Pharmacia) column equilibrated with 20 mM MES, pH 5.5, with at least 0.4 M NaCl used to prevent background binding. Fractions that tested positive for chymase activity were assessed for purity by SDS-PAGE and pooled (Fig. 2). The identity of chymase was confirmed by Western blot analysis and by N-terminal sequencing of the first 12 residues (data not shown). The S-100 chymase pool was concentrated using a Collodion vacuum dialysis unit (Schleicher and Schuell, Keene, NH) fitted with a 10 kDa membrane. After concentrating to dryness, the chymase was reconstituted with 20 mM MES, pH 5.5, to a concentration greater than 5 g/l (0.2 mM). An aliquot of 200 mM phenylmethylsulfonylfluoride (PMSF) in dimethylsulfoxide (DMSO) was added to the concentrate to at most 5% (v/v) DMSO in the final solution. Approximately 40-fold stoichiometric excess of PMSF to chymase was used.

2.3. Crystallization

PMSF-treated chymase, at a concentration of approximately 5 mg/ml, was subjected to hanging drop vapor-diffusion [24] crystallization experiments using factorial screening [25] kits (Hampton Research, Laguna Hills, CA). Preliminary crystals were obtained in a variety of polyethylene glycol (PEG) and PEG–monomethyl ether (MME) conditions. The pH of these conditions varied from 4.6 to 8.5, and salts such as ammonium sulfate, sodium chloride and sodium acetate also appear to facilitate crystallization. Two crystal forms were optimized. The first, from 20 to 30% PEG MME 2000, 0.1 M sodium acetate, pH 4.6, with 0.2 M ammonium sulfate was characterized and found to be space group C2. The cell constants are $a = 47.94$ Å, $b = 85.23$ Å, $c = 174.18$ Å, $\beta = 96.74°$. There appears to be either three or four molecules in the asymmetric unit, which corresponds to Matthews' coefficients [26] of 2.5 Å^3/Da and 1.9 Å^3/Da, respectively. These crystals diffract to at least 2.1 Å using an Raxis IV image plate system (MSC, The Woodlands, TX), with a Rigaku generator powered at 55 kV×90 mA, and have a useable X-ray lifetime of approximately 15–24 h. The second crystal form is obtained from 20 PEG 4000, 20% 2-propanol, 0.1 M sodium citrate, pH 5.6. These crystals were found to be of an orthorhombic habit, space group P212121, with cell dimensions $a = 43.93$ Å, $b = 58.16$ Å, and $c = 86.09$ Å. The Matthews' coefficient is 2.3 Å^3/Da for one molecule in the asymmetric unit. The crystals diffract to approximately 1.9 Å, and are not unusually sensitive to X-ray damage. The second crystal form provides a better opportunity for structure determination by molecular replacement. To this end, search models have been constructed from a homology model of human chymase, derived, in part, from the related rat mast cell protease II structure (pdb: 3RP2, [27]; 60% identity).

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Purification and biochemical characterization of a vacuolar serine endopeptidase induced by glucose starvation in maize roots

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An endopeptidase (designated RSIP, for root-starvation-induced protease) was purified to homogeneity from glucose-starved maize roots. The molecular mass of the enzyme was 59 kDa by SDS/PAGE under reducing conditions and 62 kDa by gel filtration on a Sephacryl S-200 column. The isoelectric point of RSIP was 4.55. The purified enzyme was stable, with no autoproteolytic activity. The enzyme activity was strongly inhibited by proteinaceous trypsin inhibitors, di-isopropylfluorophosphate, 3,4-dichloroisocoumarin and PMSF, suggesting that the enzyme is a serine protease. The maximum proteolytic activity against different protein substrates occurred at pH 6.5. With the exception of succinyl-Leu-Leu-Val-Tyr-4-methylcoumarin, no hydrolysis was detected with synthetic tryptic, chymotryptic or peptidylglutamate substrates. The determination of the cleavage sites in the oxidized B-chain of insulin showed specificity for hydrophobic residues at the P2 and P3 positions, indicating that RSIP is distinct from other previously characterized maize endopeptidases. Both subcellular fractionation and immunodetection *in situ* indicated that RSIP is localized in the vacuole of the root cells. RSIP is the first vacuolar serine endopeptidase to be identified. Glucose starvation induced RSIP: after 4 days of starvation, RSIP was estimated to constitute 80% of total endopeptidase activity in the root tip. These results suggest that RSIP is implicated in vacuolar autophagic processes triggered by carbon limitation.

INTRODUCTION

Marked changes occur when plant cells are deprived of carbohydrates [1–5]. As in natural starvation, which occurs in higher plants during senescence [6,7], shading [4,8] or after harvest [9], maize roots incubated without an exogenous carbon source substitute protein and lipid metabolism for sugar metabolism through autophagic processes [1,2,5]. There are many reports of degradation and synthesis of specific proteins during starvation. In response to starvation, plant cells decrease the activity of enzymes involved in sugar metabolism and respiration [2,5] and in nitrate reduction and assimilation [10]. In contrast, the activity of enzymes involved in fatty acid oxidation [11] and in amino acid [10] and protein degradation [12,13] increase. Moreover, the expression of various proteins [14–19] has been shown to be sugar-dependent. Thus it seems that starved plant cells are able to induce the synthesis of new or pre-existing enzymes that are involved in the response to starvation.

The co-ordinated response of plant cells to starvation suggests a fine control of proteolysis. This could be mediated by several populations of proteases implicated in various catabolic processes: (1) specific nuclear and cytosolic proteolysis, (2) organellar proteolysis and (3) lysosomal/vacuolar proteolysis (micro- and macro-autophagy or protein importation into the vacuole) (reviewed in [20,21]). Because each cellular compartment is affected by the carbon limitation, these different proteolytic systems might be involved, although to differing extents, in the cellular response to starvation. Degradation of proteins followed by amino acid catabolism and oxidation could supply carbon to the tricarboxylic cycle [10]. In a previous study we showed that starvation-induced protein degradation in the maize root tips was correlated with a transient increase in free amino acids and a rise in endopeptidase and carboxypeptidase activities [13]. The endopeptidase activities involved in the response to starvation seemed to be different from those present in non-starved tissues, indicating that the level and nature of endoproteolytic activities were controlled by the level of sugars during the starvation period [13].

Here we describe the purification to apparent homogeneity of a new vacuolar serine endopeptidase, responsible for 80% of total endopeptidase activity in maize roots starved for 4 days. The biochemical properties and the subcellular localization of the enzyme are discussed in relation to the starvation process.

EXPERIMENTAL

Chemicals

Leupeptin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), pepstatin, *p*-chloromercuribenzenesulphonate (PCMBS), phosphoramidon, 1,10-phenanthroline, PMSF, 3,4-dichloroisocoumarin, chymostatin, benzamidine, iodoacetamide, di-isopropylfluorophosphate, soybean trypsin inhibitor (STI) and egg trypsin inhibitor (ETI) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Na_2EDTA was from Merck (Darmstadt, Germany). Carrier-free $Na^{125}I$ (5.6×10^8 Bq/μg) was purchased from Amersham (Les Ulis, France). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H + L) were from Sanofi (Marnes-la-Coquette, France).

Plant materials and incubation conditions

Maize seeds (*Zea mays* L. cv. Dea; Pioneer France Maïs, Toulouse, France) were soaked for 3 h in water and germinated for 3 days on layers of wet filter paper; primary root tips 3 mm long or primary roots 3–4 cm long were then excised and either

Abbreviations used: Bz, benzoyl; Cbz, benzyloxycarbonyl; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; ETI, egg trypsin inhibitor; FITC, fluorescein isothiocyanate; Mec, 4-methylcoumarin; NA, β-naphthylamide; $NHPhNO_2$, *p*-nitroanilide; PCMBS, *p*-chloromercuribenzenesulphonate; RSIP, root-starvation-induced protease; STI, soybean trypsin inhibitor; Succ, *N*-succinyl.

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used immediately or incubated for starvation treatment. Incubation conditions were essentially as already described [5,13]. Before each renewal of incubation medium, the roots were rinsed thoroughly with pure (Milli Q) water. For protease localization experiments on stems or leaves, 3-day-old seedlings were grown for a further 4 days in pots containing a mixture of peat and vermiculite (50:50, v/v), in a phytotron at 24 °C and 80% relative humidity either in the light (photon flux density 300 μmol/s per m^2, 14 h per day) or in complete darkness (etiolated seedlings). Seedlings were watered daily with 150 ml of diluted culture medium. Stems and leaves were then harvested and frozen immediately in liquid nitrogen for protein extraction.

Preparation of crude extracts

Crude extracts of root tips (3 mm long), mature roots, germinated seeds (3 days after imbibition), coleoptiles (5 days after imbibition), stems, green leaves or etiolated leaves were used for proteolytic assay and immunodetection experiments. Fresh or frozen tissues were crushed at 4 °C in a mortar in grinding medium (0.4 ml per g fresh weight) containing 20 mM Mops, pH 7.0, 10 mM 2-mercaptoethanol and 3% (w/v) polyvinylpolypyrrolidone. The brei was transferred to a 1.5 ml microcentrifuge tube and the mortar was rinsed with grinding medium, which was then pooled with the brei. The homogenate was squeezed through a single layer of Miracloth (Calbiochem, Meudon, France) and centrifuged at 10000 *g* for 15 min. The supernatant was desalted through an Econo Pac 10-DG column (Bio-Rad) equilibrated with 10 mM Mops (pH 7.0).

Purification of the root-starvation-induced protease (RSIP)

All steps were performed at 4 °C.

Step 1

Approx. 150 g of primary roots 3–5 cm long was first incubated for 4 days in sugar-depleted medium A, and then homogenized in a Waring Blendor with 300 ml of 20 mM sodium acetate, pH 4.8, containing 10 mM 2-mercaptoethanol and 3% (w/v) polyvinylpolypyrrolidone. The homogenate was filtered through one layer of Miracloth and two layers of 50 μm nylon netting (Nylon Blutex; Tripette et Renaud, Lyon, France) and centrifuged at 20000 *g* for 20 min. The crude supernatant was used for the further purification step.

Step 2

The crude supernatant was applied to a 2.5 cm × 42 cm column of Sepharose Q Fast Flow (Pharmacia) equilibrated with 10 mM sodium acetate (pH 4.8)/2 mM 2-mercaptoethanol. The column was washed at 120 ml/h with 200 ml of the same equilibration buffer and then bound proteins were eluted at the same flow rate with 200 ml of the equilibration buffer containing 0.6 M NaCl. Fractions of 40 ml were collected and those containing the major peak of proteolytic activity were combined and concentrated in an Amicon stirred cell with a YM-30 membrane.

Step 3

The concentrated sample was applied to a Sephacryl S-200 HR (Pharmacia) gel-filtration column (2.5 cm × 70 cm) equilibrated with 50 mM Tris/HCl (pH 7.6)/50 mM NaCl and eluted at 1 ml/min. The active fractions corresponding to the major activity peak (25 ml each) were pooled and concentrated (Amicon stirred cell, YM 30 membrane).

Step 4

The concentrated fraction was diluted 1:10 with a solution of 20 mM methylpiperazine, pH 5.7, and applied to an FPLC Mono-P HR 20/5 chromatofocusing column (Pharmacia) equilibrated with 20 mM methylpiperazine, pH 5.7 as the starting buffer. Proteins were eluted at 0.5 ml/min with a pH gradient from 5.7 to 4 generated by the addition of an Ampholine solution (PBE 74; Pharmacia) diluted 1:10 in water and brought to pH 4.0 with iminoacetic acid. Active fractions of 1 ml were pooled and concentrated by ultrafiltration on membrane cones (Amicon, CF25 Centriflo system).

Step 5

The concentrated sample was brought to 50 mM potassium phosphate buffer (pH 7.0)/1.7 M $(NH_4)_2SO_4$ and applied to an FPLC Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 100 mM potassium phosphate (pH 7.0)/1.7 M $(NH_4)_2SO_4$. The column was washed with the same buffer and proteins were eluted, at 0.5 ml/min, with a 15 min linear gradient from 1.7 to 0 M $(NH_4)_2SO_4$. The proteolytic activity peak coincided exactly with only one peak of protein absorbance. The purity of the enzyme was assessed by native PAGE and SDS/PAGE [25].

Determination of molecular mass

The molecular mass of the purified enzyme was determined by gel filtration on the Sephacryl S-200 HR column, equilibrated with 50 mM Tris/HCl (pH 7.6)/100 mM NaCl and calibrated with the following proteins as standards: alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12 kDa).

Protease activity assays

Protein labelling

Casein, BSA and ovalbumin were radiolabelled with ^{125}I by the chloramine-T method [22]. The initial specific radioactivity and concentration of ^{125}I-labelled protein were approx. 10^4 c.p.m./μg and 0.5 μg/μl respectively.

Assays with iodinated proteins

For routine assays on crude extracts or chromatographic isolates, 40 μl of extract or isolate, 40 μl of pH 6.5 buffer mixture (50 mM acetic acid/50 mM Mes/100 mM Tris) [23] and 10 μl of ^{125}I-labelled protein (5000 c.p.m./μl) were incubated for 10–20 min at 37 °C. The reaction was stopped with 100 μl of 30% (w/v) trichloroacetic acid and the samples were centrifuged for 10 min at 6000 *g*; 100 μl of supernatant was mixed with 2 ml of scintillation solution and radioactivity was measured with a liquid-scintillation analyser (Tri-Carb 2000CA; Packard, Meriden, CT, U.S.A.). The linearity of casein degrading activity was checked.

Assay with synthetic substrates

N-Benzoylarginine-*p*-nitroanilide (Bz-Arg-NH-PhNO_2), *N*-benzoyltyrosine-*p*-nitroanilide (Bz-Tyr-NH-PhNO_2), *N*-benzyloxycarbonyl-Leu-Leu-Glu-β-naphthylamide (Cbz-Leu-Leu-Glu-NA), *N*-benzyloxycarbonyl-Gly-Gly-Arg-7-amido-4-methylcoumarin (Cbz-Gly-Gly-Arg-Mec), *N*-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (Succ-Ala-Ala-Phe-Mec) and *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Succ-Leu-Leu-Val-Tyr-Mec) were assayed with purified enzyme for

determination of substrate specificity. The assay mixture consisted of 10 μl of synthetic substrate (5 mM in DMSO) and 140 μl of buffer mixture (see above) with 1 μg of enzyme. After incubation for 1 h at 37 °C, the 7-amino-4-methylcoumarin or naphthylamine radicals released were measured fluorimetrically (excitation at 380 nm, emission at 460 nm; excitation at 335 nm, emission at 410 nm respectively), and the 4-nitroaniline radicals were measured spectrophotometrically at 410 nm. Measurement of endoproteolytic activity with azocasein as a substrate was performed on purified enzyme as previously described [13].

Determination of the optimum pH

The pH dependence of the proteolytic activity against iodinated casein, iodinated serum albumin, iodinated ovalbumin or azocasein as substrates was determined by using the three-component buffer mixture described above [23].

Effect of inhibitors

The effects of inhibitors on the enzymic activity were studied on purified RSIP (2 μg in 40 μl). Protease inhibitors were prepared as the following stock solutions: antipain (10 mM), leupeptin (20 mM), E-64 (10 mM), iodoacetamide (0.1 M), PCMBS (10 mM), phosphoramidon (1 mM), Na_2EDTA (0.5 M), STI and ETI (10 mM) were in water; PMSF (0.2 M) and benzamidine (0.1 M) were in ethanol; 3,4-dichloroisocoumarin (10 mM) and chymostatin (10 mM) were in DMSO; 1,10-phenanthroline (0.2 M) and pepstatin (5 mM) were in methanol; di-isopropyl-fluorophosphate (0.5 M) was in isopropanol. Inhibitors were first preincubated at ambient temperature for 15 min with RSIP before the addition of ^{125}I-labelled casein (see above), and activities were measured after 10 min reaction time. Control assays were conducted with solvent alone.

Hydrolysis of Insulin by RSIP

Oxidized B chain of bovine insulin (100 μg) was incubated at 37 °C in 10 mM potassium phosphate buffer, pH 6.5, with 1 μg of purified RSIP. At intervals, 25 μl aliquots of sample were mixed with 25 μl of buffer 1 [0.1% trifluoroacetic acid in water/acetonitrile (90:10, v/v)] and frozen in liquid nitrogen until analysis. For peptide analysis, samples were quickly thawed and immediately applied to a C_{18} reverse-phase HPLC column (Vydac, Hesperia, CA, U.S.A.) equilibrated with buffer 1. After a 10 min wash with buffer 1, peptides were separated at a flow rate of 0.7 ml/min by a 30 min linear gradient from 100% buffer 1 to 100% buffer 2 [0.1% trifluoroacetic acid in water/acetonitrile (40:60, v/v)]. Peptides were detected at 214 nm and collected. Peptides were then identified (1) by the analysis of their amino acid N-terminal sequence, with an Applied Biosystems 470A Protein Sequencer, by the method of Edman and Henschen [24], and (2) by their compositions in total amino acids. For amino acid analysis, peptides were first hydrolysed in evacuated sealed tubes with 100 μl of 6 M HCl at 110 °C for 24 h and then neutralized with 6 M NaOH. Amino acids were analysed as described previously [10].

Electrophoresis

Native PAGE and SDS/PAGE were performed with 8.5% (w/v) and 12.5% (w/v) polyacrylamide gels respectively by the procedure of Laemmli [25]. Gels were fixed, stained with Coomassie Blue and destained by standard methods.

Immunological and blotting methods

Polyclonal antibodies against maize endopeptidase (RSIP) were produced by subcutaneous injections of purified enzyme into a New Zealand white rabbit as described in [26]. Serum was stored at −80 °C in 0.02% NaN_3. RSIP-specific antibodies were purified as described in [27] and immediately used for immunoblotting determination and immunofluorescence microscopy.

For immunoblots, proteins from native PAGE or SDS/PAGE were transferred to nitrocellulose membrane (BA 85; Schleicher and Schull, Ecquevilly, France) for 1 h at 4 mA/cm^2 in a Bio-blot semi-dry system (Eurogentec, Tileman, Belgium). Blots were blocked with Tris-buffered saline containing 0.2% Tween-20 and 5% (w/v) non-fat milk powder. RSIP was detected with purified anti-RSIP antibodies plus goat anti-(rabbit IgG)–alkaline phosphatase conjugate (Sigma). Signal quantification on immunoblots was done by a scanning densitometer (Ultroscan DL, LKB). For immunoprecipitation experiments, equal caseinase activities in different crude extracts (100 c.p.m./min) or in purified protease fractions (500 c.p.m./min) were incubated for 1 h at 25 °C with increasing volumes of immune or preimmune serum. Immune complexes were incubated for 1 h at 25 °C with a 2-fold (IgG-binding) excess of Protein A–Sepharose and then centrifuged for 5 min at 10000 *g*. The caseinase activity was then measured in each supernatant fraction.

Protein determination

Proteins were quantified by the method of Bradford [28]. Bovine γ-globulin was used as the protein standard.

Subcellular fractionation

All steps were performed at 4 °C. A protocol for subcellular fractionation of sunflower coleoptiles [29] was adapted for maize root tips. Maize root tips (400; approx. 1 g fresh weight) that were either non-starved or had been starved for 24 h were gently crushed in a smooth-bottomed mortar in 5 ml of homogenization buffer [170 mM Tricine/NaOH (pH 7.5)/10 mM KCl/1 mM $MgCl_2$/1 mM EDTA/1 mM 2-mercaptoethanol/1 mM dithiothreitol/0.6 M sucrose/9 g/l BSA]. The homogenate was filtered through three layers of Miracloth and centrifuged at 600 *g* for 10 min. The supernatant was layered carefully on a discontinuous sucrose gradient. All sucrose solutions contained 1 mM EDTA, pH 7.5. The gradient consisted of 5 ml of 30%, 5 ml of 40%, 10 ml of 50% and 10 ml of 60% (w/v) sucrose. The tubes were then centrifuged at 83000 *g* for 90 min. Fractions of 1 ml were collected along the gradient, from the bottom to the top of the tube, and analysed. RSIP was immunodetected and then quantified by scanning in each fraction, after SDS/PAGE and electrotransfer to nitrocellulose membrane (see above). Marker enzymes were assayed in each fraction, and regions of the gradient corresponding to the different cell compartments were identified: fumarase (mitochondria) and phospho-enolpyruvate carboxylase (cytosol) as described in [5], NADP:glyceraldehyde-3-phosphate dehydrogenase (plastid) as in [29] and α-mannosidase (vacuole) as in [30]. The integrity of mitochondria and plastids was determined as in [31].

Microscopy and Immunofluorescence methods

Root tips were fixed 4 h at room temperature in 25 mM NaH_2PO_4/Na_2HPO_4 (pH 7.3)/100 mM sucrose/4% (w/v)

paraformaldehyde/0.5% (v/v) glutaraldehyde (EM grade). After a 30 min wash in 100 mM phosphate buffer, pH 7.2, tips were embedded in Epon. Sections (1.5 μm) were processed as described in [32]. After removal of the resin, tissues were incubated first with anti-RSIP antibodies, then with FITC-conjugated goat anti-rabbit IgG (H+L). The primary antibody was derived from the crude serum (55.6 mg/ml protein), purified serum (2.8 mg/ml protein) or purified IgG (0.68 mg/ml protein) isolated from the crude antiserum as described in [32]. Antibodies were diluted into 100 mM sodium phosphate buffer, pH 7.3, containing 200 mM NaCl, 0.1% BSA and 0.1% (v/v) Triton X-100. Control sections were incubated with preimmune serum in place of the primary antibody. Sections were counterstained with 0.01% Evans Blue in saline phosphate buffer (pH 7.3).

RESULTS

Preliminary experiments showed that, in whole maize roots as well as in maize root tips [13], the maximum increase in endoproteolytic activity occurred after 4 days of carbohydrate starvation, whereas no increase was observed in sugar-supplied (0.2 M glucose) tissues (results not shown). Thus whole roots were chosen, instead of root tips, as starting material for protein purification because their specific endoproteolytic activity was higher and large quantities of tissue could be more easily obtained.

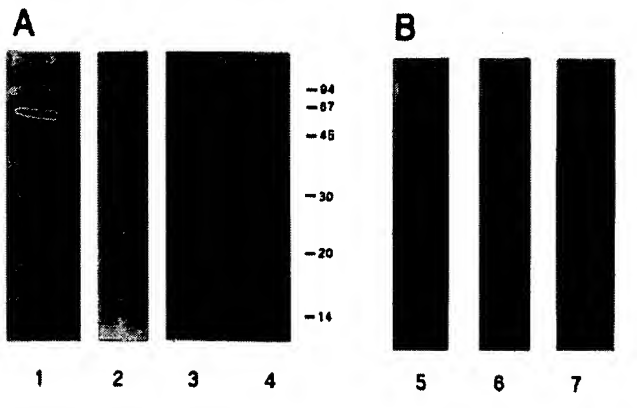

Figure 1 SDS/PAGE (A) and PAGE (B) analysis of the RSIP preparation obtained after Phenyl-Superose chromatography

The purified RSIP preparation was revealed by Coomassie Brillant Blue coloration after SDS/PAGE [12.5% (w/v) gel] (lane 3) or 8.5% (w/v) PAGE (lane 7). Purified antibodies were used to immunodetect RSIP in crude extracts of maize root (lanes 2 and 6) or in the Phenyl-Superose preparation (lanes 1 and 5). Pure RSIP (2 μg) was loaded in tracks 1, 3, 5 and 7 and 100 μg of total protein in 2 and 6. The molecular masses of markers, separated on SDS/PAGE [12.5% (w/v) gel] (lane 4), are shown in kDa.

Endopeptidase purification

The crude supernatant obtained after the first step contained a large quantity of protein (230 mg) associated with a high total endopeptidase activity (Table 1). The application of the crude supernatant to Sepharose Q Fast Flow, at pH 4.8 (step 2), resulted in the retention of the major part of total endopeptidase activity (results not shown). Although the recovery of total endopeptidase activity was low (20.5%) (Table 1), this step was necessary to remove polyphenols and microsomal fragments that were not retained on the column. After elution, the active fractions were pooled, concentrated and subjected to gel filtration on Sephacryl S-200 (step 3), which separated the major endopeptidase activity peak from the bulk of the higher-molecular-mass contaminating proteins (results not shown). The 45-fold purification factor was associated with a small loss of activity (Table 1). The endopeptidase pool was then chromatofocused on Mono P (step 4) and only one caseolytic activity peak was eluted at pH 4.55, providing an estimate of the isoelectric point of the enzyme. The purification factor at this step was 3 with no significant loss of activity (Table 1). Finally the active pool was applied to a Phenyl-Superose column and a relatively hydrophobic fraction was eluted at approx. 200 mM $(NH_4)_2SO_4$. This final step provided a purified enzyme showing a single band of protein after SDS/PAGE and native PAGE (Figure 1, lanes 3 and 7). Under denaturing conditions, the apparent molecular mass of the protease was calculated to be 59 ± 2 kDa (Figure 1). It was estimated to be 62 ± 2 kDa by gel filtration on Sephacryl S-200 (results not shown). These results indicate that the native enzyme is monomeric.

The overall purification achieved was approx. 340-fold and the apparent yield of activity was only 6% (Table 1). However, these factors are underestimates because the crude supernatant contained multiple endopeptidase activities. The five-step purification procedure typically yielded 30–50 μg of highly purified protease from 150 g (fresh weight) of maize roots. Because this endopeptidase was induced in starved material and exclusively located in roots (see below), we called it RSIP.

Immunocharacterization

Rabbit polyclonal antibodies were raised against purified RSIP. Immunoprecipitation experiments showed that the activity of

Table 1 Purification of RSIP from glucose-starved maize roots

Endopeptidase activity was determined with ^{125}I-labelled casein as a substrate. The initial specific radioactivity of ^{125}I-labelled casein was 10^4 c.p.m./μg. Activities, expressed in c.p.m./min, were measured as described in the Experimental section.

Step	Protein activity (mg)	Total activity (c.p.m./min)	Specific activity (c.p.m./min per mg)	Purification factor (fold)	Recovery (%)
Crude extract	228.6	278660	1219	1	100
Sepharose Q	38.66	57125	1478	1.2	20.5
Sephacryl S-200	0.98	54340	55450	45.5	19.5
Mono P	0.32	51273	160228	131.4	18.4
Phenyl-Superose	0.042	17276	412140	338.1	6.2

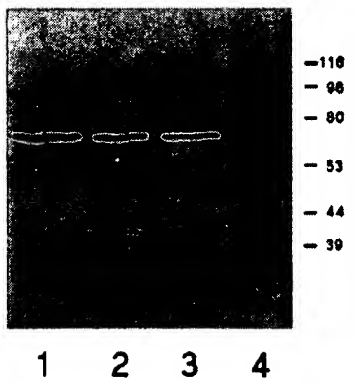

Figure 2 Determination of RSIP stability by immunoblotting

Purified RSIP (5 μg) was incubated for 3 h at 37 °C in 30 μl of a 50 mM potassium phosphate buffer (lane 1), or for 3 h (lane 2) and 6 h (lane 3) in 30 μl of a crude extract of mature maize roots starved for 4 days. Total extracts separated by SDS/PAGE [12.5% (w/v) gel] under reducing conditions followed by immunodetection with purified anti-RSIP antibodies. The molecular masses of prestained molecular mass markers (lane 4) are shown in kDa.

purified RSIP was 90% and 98% inhibited on addition of 10 and 20 μl of crude antiserum respectively (results not shown). Further purification of antibodies by antigen-immobilized affinity [27] was used to increase their specificity for RSIP. The purified antibody was found to be specific for RSIP because it gave a single band on immunoblots against both the crude extract and the purified enzyme in SDS/PAGE (Figure 1, lanes 1 and 2) as well as in native PAGE (Figure 1, lanes 5 and 6).

Biochemical characterization

Stability of the enzyme

The stability of the purified RSIP was tested under various storage conditions. Enzyme activity at pH 7.0 was stable: after 1 h the loss of activity was only 25% at 50 °C, whereas no change was observed at 25 or 4 °C (results not shown). After 5 days at −20 °C, the conservation of RSIP was improved in the presence of 5 mM 2-mercaptoethanol and 5% (v/v) glycerol. Under these conditions activity was not significantly modified after multiple cycles of freezing and thawing (results not shown). The positive effect of 2-mercaptoethanol suggested the presence of an important thiol group in the protease. The effect of pH on the storage stability of RSIP was also estimated after 5 days of conservation at −20 °C (results not shown); it seems that the enzyme was stable at a large range of pH values (5–9) but was less active after conservation under more acidic conditions (pH 3). No autoproteolytic degradation was observed when purified enzyme was incubated at 37 °C (Figure 2, lane 1). However, when RSIP was incubated at 37 °C in the presence of a crude extract of maize roots starved for 4 days, degradation peptides were detected on immunoblots (Figure 2, lanes 2 and 3). These results suggest that in a crude extract RSIP was degraded by other proteases and not by autoproteolysis.

Substrate specificity and optimum pH

The activity of the protease was assayed with several ^{125}I-labelled proteins (Table 2). RSIP degraded the three iodinated protein substrates; the activity was the highest with BSA and casein. Azocasein was also found to be a good substrate for pure RSIP. However, RSIP exhibited little or no activity against various synthetic substrates; only Succ-Leu-Leu-Val-Tyr-Mec was hydrolysed but at a low rate (Table 2).

The purified protease was maximally active at pH 6.5–7.0

Table 2 Hydrolysis of different protein or synthetic substrates

Assays were performed as described in the Experimental section. Abbreviation: n.d., not detected.

Substrate	Concentration	Activity
^{125}I-labelled protein substrate	(c.p.m. per assay)	(c.p.m./min per μg of protein)
Casein	50000	410
BSA	50000	580
Ovalbumin	50000	160
Synthetic peptide substrate	(mM)	(pmol/min per μg of protein)
Bz-Arg-NHPhNO$_2$	0.3	n.d.
Bz-Tyr-NHPhNO$_2$	0.3	n.d.
Cbz-Leu-Leu-Glu-NA	0.3	n.d.
Cbz-Gly-Gly-Arg-Mec	0.3	n.d.
Succ-Ala-Ala-Phe-Mec	0.3	n.d.
Succ-Leu-Leu-Val-Tyr-Mec	0.3	1.1
Azoprotein substrate	(mg/ml)	(units/mg of protein)
Azocasein	1.8	2.8

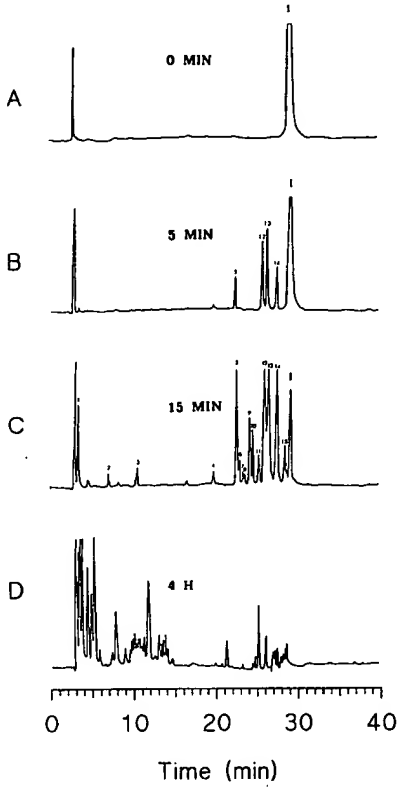

Figure 3 Kinetics of degradation of the insulin B chain by RSIP

The insulin B chain (100 μg) was incubated in a 10 mM potassium phosphate buffer (pH 6.5) with 1 μg of pure RSIP in a final volume of 120 μl. At the times indicated, 0 min (**A**), 5 min (**B**), 15 min (**C**) and 4 h (**D**), aliquots of the reaction mixture were removed and peptides were analysed on a HPLC reverse-phase column by monitoring absorbance at 214 nm, as described in the Experimental section. Peptides from 1 to 15 were clearly separated from the insulin B chain peak (I).

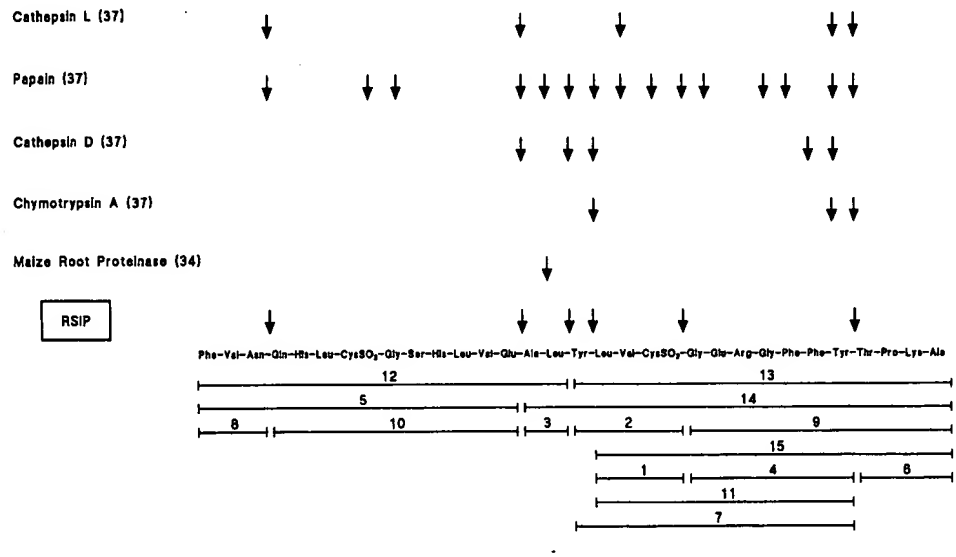

Figure 4 Localization of the RSIP cleavage sites on the insulin B chain

Peptides (numbered 1 to 15) obtained from insulin B chain digestion and separation on a reverse-phase HPLC column (Figure 3) were identified by analysis of their N-terminal sequence and by their amino acid compositions. Arrows indicate the different cleavage sites deduced from the sequences of peptides cleaved by RSIP. A comparison of RSIP cleavage sites with those of other, well-characterized, endopeptidases [34,37] is also shown.

against casein, BSA and ovalbumin (results not shown). Azocasein was maximally degraded at pH 6.1, which might be due to some modifying effect of its sulphanilamide chromophore [33].

Specificity of the cleavage site

The specificity of cleavage site was studied by following the action of the purified protease on the oxidized insulin B chain. Chromatographic analysis of peptide fragments released during digestion showed that insulin was hydrolysed rapidly (Figures 3A, 3B and 3C). With a substrate-to-enzyme mass ratio of 100:1, approx. 80% of the insulin was hydrolysed within 15 min and 100% within 30 min. After 5 min of hydrolysis, four major peptides were found (numbered 5, 12, 13 and 14) (Figure 3B). From 5 to 15 min, secondary hydrolysis products were generated besides the former major peptides (numbered from 1 to 15) (Figure 3C). After several hours, less specific cleavage sites occurred, providing a large number of small peptides (Figure 3D). The abundance of generated peptides was an indication of the large domain of activity of RSIP. However, the identification of the peptides released during short-term hydrolysis clearly demonstrated a preference for cleavage sites (Figure 4) with hydrophobic amino acids located at the P2 and, to a lesser extent, the P3 positions : Phe^{1}-Val^{2}, Leu^{11}-Val^{12}, [Glu^{13}]-Ala^{14}, Ala^{14}-Leu^{15}, Leu^{17}-Val^{18} and Phe^{24}-Phe^{25}.

Table 3 Effect of various inhibitors on protease activity

Enzyme (2 μg) was preincubated with different inhibitors for 15 min before the addition of ^{125}I-labelled casein. Activity is expressed as a percentage of the control (100% of activity represented 9000 c.p.m. for 10 min reaction time). Control assays were performed with solvent alone. Abbreviations: DFP, di-isopropylfluorophosphate; 3,4DCI, 3,4-dichloroisocoumarin.

	Inhibitor concentration (mM)	Activity (% of control)
Control		100
PMSF	5	3
DFP	5	12
3,4DCI	0.1	11
STI	0.01	0
ETI	0.01	8
Benzamidine	5	95
E-64	0.5	108
N-Ethylmaleimide	5	91
Iodoacetamide	1	82
PCMBS	0.5	28
Chymostatin	0.1	2
Leupeptin	0.2	90
EDTA	10	93
1,10-Phenanthroline	10	88
Phosphoramidon	0.01	92
Pepstatin	0.05	101

Effect of inhibitors

The inhibitor study was intended to determine the mechanistic class of RSIP. The effect of various compounds on the activity of the purified protease is shown in Table 3. All the serine protease inhibitors tested were strong inhibitors of caseolytic activity (90–100% inhibition), except benzamidine, which was practically ineffective. The strong inhibition of the purified enzyme by PMSF, which was not reversed by L-cysteine or 2-mercaptoethanol in the assay medium (results not shown), confirmed the assignment of RSIP to the serine protease class. Proteinaceous inhibitors, STI and ETI, and the microbial inhibitor chymostatin were also efficient inhibitors of endopeptidase activity. In contrast, cysteine protease inhibitors such as E-64, *N*-ethylmaleimide, iodoacetamide or leupeptin had no

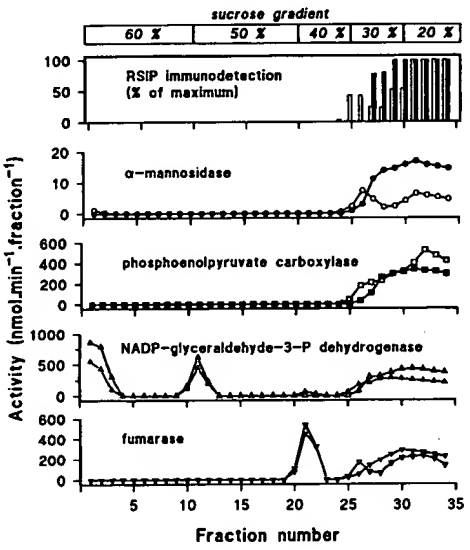

Figure 5 Determination of the RSIP localization of by subcellular fractionation

The different compartments were separated by centrifugation on a discontinuous sucrose gradient, and marker enzyme activities were measured along the gradient. The densities of the different sucrose layers are indicated at the top. Maize root tips were non-starved (open symbols) or starved for 24 h (filled symbols). Fractions of the gradient were also immunoblotted for RSIP immunolocalization (open bars, non-starved; filled bars, starved for 24 h). The absolute value of 110% in fractions from starved root tips is 5-fold that from non-starved root tips.

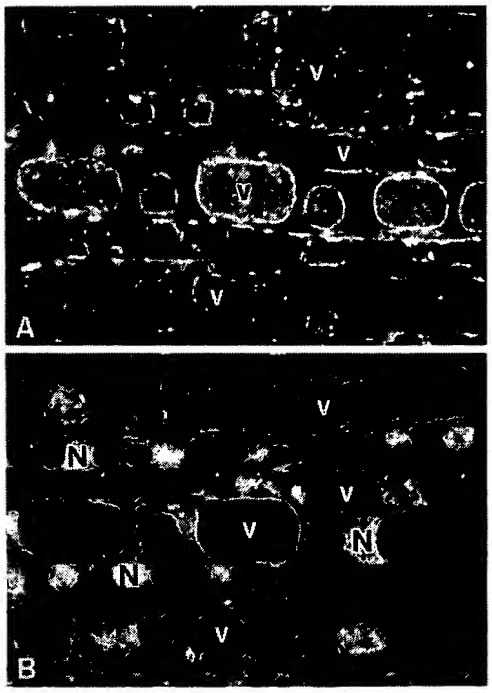

Figure 6 Intracellular immunolocalization of RSIP in a maize root tip

Longitudinal section of a maize root tip starved for 24 h at the boundary between the cortex and the medulla, approx. 700 μm above the apical meristem. (**A**) Test section incubated with purified anti-RSIP antibodies. The tonoplast is generally underlined by peripheral fluorescent precipitates, and the sap of the largest vacuoles (V) is strongly labelled. Unlabelled structures are counterstained with Evans Blue; their red fluorescence is considerably weaker than the FITC labelling and is poorly visible or invisible in black and white. (**B**) Control section corresponding to (**A**) incubated with preimmune serum instead the purified antibodies. Vacuoles are not labelled and the counterstained nuclei (N) are clearly visible. Magnification ×590.

marked effect on RSIP activity. However, PCMBS caused 72% inhibition, suggesting the presence of at least one essential thiol group in the protease. Metalloprotease inhibitors (1,10-phenanthroline, EDTA and phosphoramidon) and aspartic protease inhibitor (pepstatin) did not inhibit RSIP activity.

Subcellular localization of RSIP

The subcellular localization of the enzyme in the root tips, either starved for 24 h or non-starved, was studied by discontinuous density gradient centrifugation. The locations of RSIP and the different marker enzymes along the gradient are shown in Figure 5. The recovery of the various enzyme activities on the gradient, compared with crude extract [5], was close to 100%. Immunodetection of RSIP showed that it was located at the top of the gradient, in the 20 and 30% sucrose layers. With non-starved material RSIP appeared as a double peak and the immunosignal was one-quarter to one-fifth as strong as that from starved root tips. Comparison with marker enzyme patterns clearly showed that RSIP was not associated with plastidial or mitochondrial compartments. Indeed, three populations of plastid were located along the sucrose gradient: in the pellet, at the 50–60% interface and at the top of the gradient (Figure 5). Similar organellar integrity (results not shown) was observed in starved and non-starved conditions: 85% for the pellet and 60% at the 50–60% interface, whereas NADP:glyceraldehyde-3-phosphate dehydrogenase activity in the top of the gradient could be attributed to broken plastids (5% integrity). Similarly, in non-starved maize root tips (Figure 5), three different peaks of fumarase activity were detected. The integrity of the mitochondria was 91% at the 40–50% interface, declined to 30% at the 30–40% interface, and was only 5% at the top of the gradient. After 24 h of starvation the 30–40% peak became undetectable. As with plastids, the fumarase activity peak at the top of the gradient corresponded to broken mitochondria.

Both phosphoenolpyruvate carboxylase (cytosol marker) and α-mannosidase (vacuole marker) activities were located at the top of the gradient, with either starved or non-starved material (Figure 5). Under these extraction conditions, vacuoles were broken and their contents remained in the supernatant fraction. The RSIP pattern resembles the α-mannosidase pattern more than the phosphoenolpyruvate carboxylase one; particularly, both RSIP and α-mannosidase exhibit a double peak with non-starved material, and the same increase in activity occurred in starved root tips. However, these similarities were not convincing enough to allocate RSIP to the vacuole rather than to the cytosol. Thus the detection of RSIP *in situ* by immunofluorescence was necessary to determine whether the enzyme was exclusively localized in the vacuole or in the cytosol.

RSIP was weakly detected in unstarved excised root tips. However, after 24 h of starvation, RSIP was clearly expressed from the excision plane (at 3 mm from the tip) to the area of cell lengthening located between 0.5 and 1 mm above the apical meristem (Figure 6). In this area the tonoplast was underlined by peripheral precipitates, and the vacuole sap (not the cytosol) was strongly labelled in differentiating vascular tissues and in epidermis. Young vacuoles from meristematic cells at the root apex were not labelled. Cells close to the excision plane were degenerating and large zones were strongly labelled. After 48 h of starvation, RSIP was expressed in the meristematic cells of the

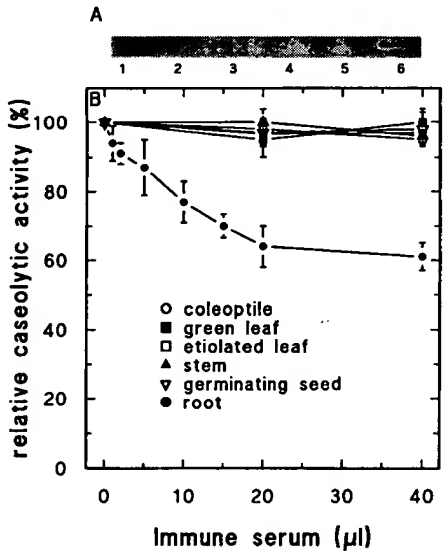

Figure 7 Distribution of RSIP in various organs of maize whole plants

(**A**) Crude extracts (100 μg of proteins) prepared from coleoptile (1), green leaf (2), etiolated leaf (3), stem (4), germinating seed (5) and roots 3 cm long (6) from maize plants were analysed by SDS/PAGE followed by immunoblotting with the purified anti-RSIP antibodies. (**B**) Equal amounts of caseolytic activity obtained from different maize tissues (100 c.p.m./min) were mixed with anti-RSIP antiserum.

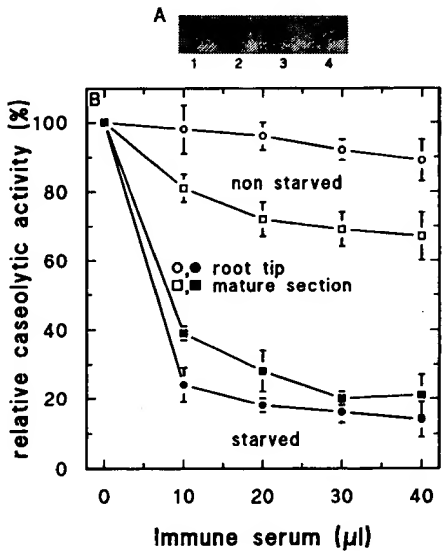

Figure 8 Distribution of RSIP in non-starved maize roots and in roots starved for 4 days

Equal amounts of caseolytic activity (100 c.p.m./min) were analysed either by SDS/PAGE followed by immunoblotting with purified anti-RSIP antibodies (**A**) or by immunoprecipitation tests with crude antiserum (**B**). Root tips: non-starved (1, ○) and starved (2, ●); mature roots: non-starved (3, □) and starved (4, ■).

apex, the vacuoles of which had tonoplast underlined by sharp fluorescent labelling (results not shown).

RSIP abundance in different maize tissues

To determine whether the same endopeptidase polypeptide was expressed in different maize cell types, proteins in crude extracts of roots, stems, germinated seeds (minus coleoptile and primary root), coleoptiles, green leaves and etiolated leaves were separated by SDS/PAGE, transferred to nitrocellulose membrane and probed with purified antibody. When equal amounts of protein were loaded on a gel, only root tissues exibited an immunosignal (Figure 7A). In addition, no inhibition of caseolytic activity was observed when the crude extracts of the different tissues were subjected to immunoprecipitation tests with anti-RSIP antibodies (Figure 7B). These results suggest that RSIP was specifically localized in roots and absent or undetectable in aerial tissues or in germinating seeds.

RSIP root distribution under starved and non-starved conditions

The exclusive occurrence of RSIP in roots raises the question of its distribution as a function of the differentiation state of the cells before and after starvation. Preliminary results had shown that when mature roots 3–4 cm long were subjected to glucose starvation, increased endoproteolytic activity was observed in both the mature and the meristematic regions of the root (results not shown). Thus crude extracts of starved and non-starved root tips and more mature subapical segments were subjected to SDS/PAGE and RSIP immunodetection experiments. When equal amounts of endopeptidase activity were separated, RSIP could be detected in both parts of the root (Figure 8A, lanes 1 and 3). Signal intensity was stronger in the mature section than in the root tip. After 4 days of starvation, the intensity was higher in both parts of the root, showing an enrichment of RSIP content (Figure 8A, lanes 2 and 4). When equal amounts of protein were separated, RSIP signal was barely detectable in non-starved root tips, whereas it was clearly visible in root tips starved for 4 days (results not shown). The same overexpression was observed, although to a smaller extent, in mature segments (results not shown). Immunoprecipitation tests of equal caseolytic activity (100 c.p.m./min) (Figure 8B) showed that almost 80 % inhibition was obtained in both root tips and mature sections after 4 days of starvation, whereas only 10–30 % of inhibition was obtained in non-starved tissues (Figure 8B). When mature roots and root tips were incubated for 4 days in the presence of 0.2 M glucose, no significant changes in the immunosignal intensity or in the percentage inhibition of caseolytic activity were observed (results not shown) These results show that RSIP (1) is a constitutive endopeptidase, (2) occurs mainly in differentiated cells and at a basal level in the root apex, (3) is overexpressed in root tissues subjected to starvation treatment, and (4) is the major endopeptidase (as measured by its caseolytic activity) present in glucose-starved root tissues.

DISCUSSION

In a previous report [13] we described the occurrence of increasing proteolytic activities in crude extracts of excised maize root tips subjected to glucose starvation. Here we describe the purification and the characterization of the major endopeptidase, RSIP, implicated in this starvation response. RSIP has been purified in four chromatographic steps (Table 1) and used for the production of polyclonal antibodies. It was shown to be a 60 kDa monomeric endopeptidase with a pI of 4.55. RSIP was strongly sensitive to serine protease inhibitors and insensitive to cysteine protease, aspartic or metalloprotease inhibitors (Table 3), and thus can be classified as a serine protease [34]. However, the stabilizing effect

of 2-mercaptoethanol on RSIP and the partial inhibition of the protease by PCMBS (Table 3) suggest the possible involvement of a cysteine residue close to the active site, or the presence of an essential thiol group in the protein structure [34].

Mature maize roots have already been reported to contain at least two endopeptidase activities, called proteases I and II [33]. Protease II did not degrade azocasein, was inhibited by serine and thiol protease inhibitors, and thus clearly differs from RSIP. Protease I, recently renamed MRP (maize root protease) [35], was also found to be a serine protease. However, it degraded azocasein optimally at pH 9–10, had a molecular mass of 54 kDa and an isoelectric point between 5 and 6 [33], which all distinguish MRP from RSIP. Moreover the study of the primary cleavage site of the oxidized B chain of insulin showed that the major cleavage site for MRP requires an alanine residue in the P1 position [35], whereas the hydrolytic attack catalysed by RSIP is independent of the nature of amino acids P1 and P1′ (Figure 4). Thus, as previously suggested in [13], RSIP is a newly identified maize root protease.

The analysis of the cleavage sites (Figure 3 and 4) showed that the presence of hydrophobic amino acids at the P2 and, to a smaller extent, P3 positions seems essential for cleavage by RSIP. However, RSIP had little activity against chromogenic substrates such as Succ-Leu-Leu-Val-Tyr-Mec and was inactive against Cbz-Leu-Leu-Glu-NA and Succ-Ala-Ala-Phe-Mec, which also possess hydrophobic amino acids at the P2 and P3 positions (Table 2). Thus it is likely that other parameters, such the structural conformation or the length of the polypeptide chain, play a role in the recognition mechanism of the cleavage site. Such substrate specificity clearly distinguishes RSIP from other serine endopeptidases, such as chymotrypsin (Figure 4), elastase or trypsin, which are active against chromogenic substrates of low molecular mass [36] and are selective for amino acids at the P1 position [37], and links it with the second major group of serine endopeptidases, for which amino acids at positions away from the scissile bond are equally important for cleavage-site specificity [37]. RSIP shares several cleavage sites with lysosomal cysteine or aspartic endopeptidases such as papain, cathepsin L or cathepsin D (Figure 4), which require hydrophobic amino acids at the P2 (and possibly P3) position [38].

With casein, BSA or ovalbumin as substrates, the activity of RSIP was maximal between pH 6 and 7. Although some plant serine proteases with acidic pH optima have been reported [39], an acidic pH optimum is a characteristic more commonly associated with cysteine proteases [38,39]. The high activity of RSIP at pH 5.5 (80% of its optimum) is consistent with its subcellular localization in the vacuole, where the pH is estimated to be approx. 5.5 [40]. Indeed, the vacuolar location of RSIP has been established by both cell fractionation and immunocytochemical studies with anti-RSIP antibodies (Figures 5 and 6). Such localization was unexpected because, so far, all the endopeptidases characterized and localized in the vacuolar/lysosomal compartment were of either a cysteine or an aspartic type, but not of a serine type, protease [38,41]. RSIP is thus the first vacuolar serine endopeptidase to be identified.

In plants, the expression of vacuolar proteases has been related to developmental processes such as seed germination, which involves the mobilization of storage protein [42] or the maturation of vacuolar protein [43].During senescence in leaves, it has been shown that proteins are degraded and proteolytic activities increase [6,7,41,44,45], but this increase has never been proved to be related to vacuolar proteolysis [7,46]. In the carbon-starved plant cell, proteins are intensively degraded through autophagic processes [2,5,9,10], and it was suggested that vacuolar proteolysis might supply amino acids for protein synthesis or energy production [13]. The present study shows that a vacuolar endopeptidase, specifically present in the roots of maize, is strongly induced in response to sugar starvation (Figures 7 and 8). As already proposed by Vierstra [21], this confirms that plant cells, like mammalian [47] and yeast [48] cells, are able to respond to drastic environmental changes or stress, i.e. carbon starvation, through enhanced vacuolar protein breakdown.

The role of RSIP in carbon-starved maize roots is probably to take part in the general catabolism that characterizes starved cells. Indeed, we previously showed that, in maize root tips, starvation can be divided into three phases: (1) acclimation, (2) survival and (3) cell disorganization [10]. Starvation for 4 days corresponds to the end of the survival phase during which the cell structures and the protein are non-selectively subjected to degradation. During this phase, root cells are probably the site of autophagic uptake of cytoplasm into the vacuoles, as observed in nutritionally stressed yeast [49] or animal cells [47]. Therefore RSIP might be the major protease involved in the degradation of cellular proteins because it is the major vacuolar endopeptidase (80% of the total activity *in vitro*). This point of view is reinforced by the low specificity of RSIP. Indeed, because the frequency of occurrence of hydrophobic dipeptide sequences is relatively high in proteins, extensive fragmentation of these proteins during glucose starvation would be expected in the presence of RSIP, and the carboxypeptidase activities, which increase during starvation [13], could act concomitantly with the endopeptidase to complete the hydrolysis of peptide chains.

RSIP was shown to be markedly induced in glucose-starved roots. The role of sugars in the regulation of gene expression has been extensively described in prokaryotic and yeast systems (see [50,51] and references therein). In plants, such studies are more recent [52], and some work has shown that the gene expression of enzymes involved in vegetative storage protein [53] or sucrose [16] metabolism, glyoxylic acid cycle [54] and photosynthesis [55] can be regulated by sugars and/or their derivatives (ester phosphates). In good agreement with our previous results [13], RSIP expression could also be regulated by intracellular sugar/phospho-sugar content. This hypothesis is currently under investigation in our laboratory.

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Subcellular Localization of CAF

In careful studies by Reville *et al.*,[27] most of the calcium-activated proteolytic activity in skeletal muscle was shown to be in the soluble fraction. Because of the difficulties of homogenizing muscle without disrupting intracellular organelles, I decided to examine a soft tissue for which fractionation procedures have been well established. Unfortunately, as with bovine heart, the amount of inhibitor relative to CAF in liver is sufficiently large so as to make detection of the protease very difficult.[6] Therefore, rat kidney was chosen as the tissue for these studies. From these experiments, it was found that approximately one half of the calcium-activated protelytic activity was in the cytoplasmic fraction, and the balance was in the microsomal fraction.[7] None could be detected in any other subcellular fraction. Moreover, extraction of the microsomes with 0.2 *M* NaCl failed to solubilize any activity.

Clearly, more sophisticated techniques, such as the use of fluorescent antibodies to CAF, will be needed to clarify this issue. Hopefully, this knowledge will also be a clue to the role of CAF in the cell.

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[50] Proteases in *Escherichia coli*

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Although more has been learned about the regulation and selectivity of intracellular protein degradation in *Escherichia coli* than in any other cell,[1-3] the proteolytic enzymes in these organisms have only been re-

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cently defined.[4-7] A detailed study of these enzymes is essential for elucidating the pathways of degradation of normal and abnormal proteins,[1,2] and the acceleration of protein breakdown when cells enter nutritionally poor conditions.[1] In these processes, cell proteins are digested completely to amino acids, and recent studies have indicated the sequential involvement of one or more endoproteases and multiple peptidases.[8-10] In addition, proteolytic enzymes must also play a key role in other important cellular processes involving only limited proteolytic cleavages, such as the maturational processing of secretory and membrane proteins,[11,12] phage morphogenesis,[3,13] breakdown of colicins,[14,15] and inactivation of certain regulatory proteins.[3,5,16,17] Finally, peptidases and perhaps proteinases are also required for the bacteria to utilize exogenous peptides,[13] although *E. coli*, unlike many gram-positive organisms, do not secrete proteases and therefore can not use polypeptides as a source of amino acids.

Proteases in E. coli

There are a growing number of reports in the literature concerning the isolation of proteases and peptidases from *E. coli*. The so-called protease I and II were identified first and purified by their ability to hydrolyze chromogenic ester substrates of chymotrypsin (e.g., *N*-acetyl-phenylalanine β-naphthyl ester) and trypsin (e.g., *N*-benzoyl-DL-arginine-*p*-nitroanilide) respectively.[18,19] These esterases are sensi-

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tive to diisopropyl fluorophosphate (DFP), but unlike the pancreatic proteases or the *E. coli* proteases described below, these enzymes show very little or no ability to hydrolyze protein substrates.[19-21] Furthermore, mutants lacking these two enzymes do not show any decreased ability to degrade abnormal or normal proteins to free amino acids.[10,21,22] Thus these esterases do not appear to play an essential role in these degradative processes. An additional serine esterase has been purified recently from *E. coli* using *N*-carbobenzoxy-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide, a synthetic substrate of subtilisin.[23] Its properties and amino acid composition are similar to those of an intracellular serine protease found in several species of *Bacillus*. However, its ability to hydrolyze proteins has not yet been established.

A number of proteolytic activities have been isolated that are capable of hydrolyzing polypeptides to acid-soluble material. Regnier and Thang[24,25] partially purified, from *E. coli* extracts, an activity that digests casein and that they call protease A to distinguish it from two other proteolytic peaks (B and C), which were not defined further. Subsequent studies[7] showed that protease A actually is a mixture of three different enzymes, two active against casein (Mi and Fa) and one against insulin (Pi). In addition, Cheng and Zipser[4] have purified an enzyme, named protease III, by using as a substrate, the amino-terminal fragments of β-galactosidase ("auto-α"). These polypeptides (MW ~ 7000) can be generated by autoclaving β-galactosidase and can be assayed rapidly and with sensitivity by using *in vitro* complementation.[26] This assay has been used extensively to follow the degradation of nonsense fragments of β-galactosidase in intact cells.[22,26-28] However, protease III is not responsible for this degradative process *in vivo*, since mutants lacking this protease degrade such nonsense fragments as rapidly as wild-type cells.[29] As will be discussed, protease III appears identical to the periplasmic insulin-degrading enzyme, named protease Pi, that was isolated by Swamy and Goldberg[7] by a very different approach (see following discussion). After this review was completed, Regnier isolated a casein-degrading enzyme from the outer membrane of *E. coli* which was named protease IV.[29a,29b]

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A systematic attempt to define the proteolytic enzymes in *E. coli* has been carried out recently by Swamy and Goldberg.[7] These workers have isolated eight soluble enzymes that hydrolyze radioactively labeled globin, casein, or insulin. All these enzymes have been purified almost to homogeneity by methods to be described. Their physical and chemical properties indicate that they are distinct endoproteases.[7] The six that degrade globin or casein are serine proteases. The other two enzymes are inactive against these substrates but rapidly hydrolyze smaller polypeptides, such as insulin or glucagon. One of these serine proteases is of particular interest, since it degrades globin and casein only in the presence of ATP. This requirement for a nucleoside triphosphate can explain the stimulation of proteolysis by ATP in crude *E. coli* extracts[30] and the energy requirement for protein breakdown in intact cells.[1,27,31,32]

For most of the proteases described thus far, clear physiological functions have not been established. One notable exception is the *recA* gene product, a protein with multiple enzymatic activities that has been purified to homogeneity by Roberts *et al.*[5] This 40,000-MW protein not only is essential for genetic recombination,[33] but also catalyzes the endoproteolytic inactivation of the phage λ-repressor during lysogenic induction of the prophage.[5,34] The purified *recA* protein cleaves the λ-repressor in the presence of ATP and a polynucleotide,[35] and also inactivates in a similar reaction the repressor of the *recA* gene (the *lexA* gene product).[17] This enzyme, however, does not digest other commonly employed protein substrates (e.g., casein or globin). Thus the *recA* product clearly differs from the ATP-dependent protease (protease La), which is very active against such polypeptides.[7-9]

A variety of observations indicate that protease La catalyzes the rate-limiting step in the breakdown of abnormal proteins *in vivo*. Studies with intact cells indicated that the initial endoproteolytic steps in this process require metabolic energy,[27] apparently because of the ATP requirement of protease La. Genetic studies have shown that mutants in *lon* gene (also called *capR* and *deg*) lead to a reduced capacity of the cells to degrade abnormal proteins *in vivo*.[28,36] Very recently, the product of the *lon* gene has been purified to near homogeneity[37] and the purified protein shown to

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be an ATP-dependent protease.[37a,37b] This activity appears identical to protease La in many physical and chemical properties.[37b] Furthermore, the content of protease La (but no other *E. coli* protease) is severalfold greater in bacterial strains in which the *lon* gene is cloned on plasmid, RGC 121/pJMC40.[37b]

In bacteria as in mammalian cells, secreted and membrane proteins are synthesized as larger precursors and then undergo limited proteolytic processing.[11,12] The mechanisms of this process in *E. coli* remain controversial. Recently Zwizinski and Wickner purified an activity from the *E. coli* membrane that they termed "leader peptidase" and that catalyzes the processing of the procoat protein of phage M13.[38] Inner membrane fractions also appear active in the maturational processing of alkaline phosphatase, a periplasmic enzyme.[39] However, it is presently unclear whether one or more "signal peptidases" exist.

One important clue to the function of the other proteases is their subcellular distribution.[39a] A number of these enzymes are found within the cytoplasm (where intracellular protein degradation probably occurs), and two are localized in the periplasmic region.[7] One casein-degrading enzyme is found in both locations. The periplasmic localization suggests a possible role in the degradation of surface proteins, of signal peptides, or of exogenous polypeptides (e.g., colicins). In addition, there is appreciable proteolytic activity associated with the cell membrane even after extensive washing.[8,40,41] One protease has been demonstrated recently on the outer membrane.[29a,29b] Furthermore, in gently lysed cell homogenates, most of the ATP-stimulated protease activity can be found in the membrane fraction.[8] The relationship between the membrane-associated ATP-stimulated protease found in gently lysed extracts and the soluble ATP-dependent protease (La) remains to elucidated.

Peptidases in E. coli

E. coli can utilize exogenous peptides as a source of essential amino acids or as metabolizable substrates, and these bacteria contain specific transport systems for the uptake of small peptides.[42,43] Specific peptidases

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are essential for growth on defined di- or tripeptides, and this requirement has allowed the selection of mutants lacking the enzymes.[13,44] In addition, certain of these intracellular peptidases seem to play an important role in the complete degradation of intracellular proteins. In a strain lacking multiple peptidases, protein degradation during starvation for a carbon source occurs more slowly than in the wild type.[10] The slower breakdown of proteins in these strains results from the inability of these bacteria to convert peptides to free amino acids. Voellmy *et al.*[9] have also found that the crude extracts from a multiple peptidase-deficient strain hydrolyzed ^{35}S-labeled *E. coli* proteins only to large acid-soluble peptides, whereas the extracts from wild-type cells hydrolyzed them to free amino acids. Thus these peptidases appear to function in the final steps of protein degradation following the action of endoproteases.

A number of peptide hydrolases have also been isolated and purified from *E. coli*, including several aminopeptidases,[45–50] three dipeptidases,[51–53] and a dipeptidyl carboxypeptidase.[54] In addition, several peptidase-deficient mutants have been isolated in *E. coli* and in the closely related bacteria *Salmonella typhimurium*.[45,55] Most of these mutations have been mapped and their genetics have been reviewed by Miller.[13] The purification and properties of these various peptidases will not be discussed.

Nomenclature of Proteases

A novel nomenclature for the eight soluble proteases has been suggested by Swamy and Goldberg.[6,7] The obvious, overworked approaches would involve numerical or alphabetical designations. However, such names have been used already to refer to enzymes that do not hydrolyze proteins at significant rates (e.g., protease I and II), or to mixtures of several enzymes (proteases A, B, C) or to peptidases (A, B, D, M, N, P, Q) that are inactive against proteins. Since the two enzymes that degrade insulin (or similar-sized polypeptides) have distinct compartmentalizations in the cell, we suggest the name Pi for the periplasmic insulin-degrading

[44] C. G. Miller and G. Schwartz, *J. Bacteriol.* **135**, 603 (1978).
[45] S. Sarid, A. Berger, and E. Katchalski, *J. Biol. Chem.* **237**, 2207 (1962).
[46] C. S. Tsai and A. T. Matheson, *Can. J. Biochem.* **43**, 1643 (1965).
[47] V. M. Vogt, *J. Biol. Chem.* **245**, 4760 (1970).
[48] A. Yaron and A. Berger, this series, Vol. 19, p. 521.
[49] C. Lazdunski, J. Busuttil, and A. Lazdunski, *Eur. J. Biochem.* **60**, 363 (1975).
[50] L. M. Yang and R. Somerville, *Biochim. Biophys. Acta* **445**, 406 (1976).
[51] E. Haley, *J. Biol. Chem.* **243**, 5748 (1968).
[52] E. Patterson, J. S. Gatmaitan, and S. Hayman, *Biochemistry* **12**, 3701 (1973).
[53] J. L. Brown, *J. Biol. Chem.* **248**, 409 (1973).
[54] A. Yaron, D. Mlyner, and A. Berger, *Biochem. Biophys. Res. Commun.* **47**, 897 (1972).
[55] C. G. Miller and K. Mackinnon, *J. Bacteriol.* **120**, 355 (1974).

enzyme, and Ci for the cytoplasmic insulin-degrading enzyme.[39a] For the proteases that degrade globin and casein, such a simple classification is not useful. Swamy and Goldberg[6,7] therefore introduced an easily remembered designation for these enzymes: Do, Re, Mi, Fa, So, La, in the order of their elution from DEAE-cellulose. One advantage of this nomenclature is that the names can be readily applied for the names of the genetic loci that code for these proteases. When more precise information is available about their physiological or chemical specificities, a more descriptive nomenclature will be possible.

Isolation and Properties of the Proteases

Buffers

The pH of all Tris-HCl buffers is adjusted at 4°C.

Buffer A: 50 m*M* Tris-HCl (pH 7.8)–10 m*M* $MgCl_2$–200 m*M* KCl
Buffer B: 10 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$
Buffer C: 50 m*M* Tris-HCl (pH 8.1)–10 m*M* $MgCl_2$
Buffer D: 50 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$–100 m*M* NaCl
Buffer E: 10 m*M* Sodium acetate, pH 5.6
Buffer F: 10 m*M* Tris-HCl, pH 8.4
Buffer G: 25 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$–50 m*M* NaCl
Buffer H: 10 m*M* Sodium acetate (pH 5.2)–2.5 m*M* $MgCl_2$
Buffer I: 20 m*M* Potassium phosphate, pH 7.4
Buffer J: 50 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$–50 m*M* NaCl
Buffer K: 10 m*M* Sodium acetate (pH 5.0)–2.5 m*M* $MgCl_2$
Buffer L: 10 m*M* Tris-HCl (pH 8.4)–1 m*M* cysteine–20 m*M* NaCl–5 m*M* $MgCl_2$
Buffer M: 10 m*M* Tris-HCl (pH 8.0)–5 m*M* $MgCl_2$–10 m*M* 2-mercaptoethanol
Buffer N: 10 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$–100 m*M* NaCl
Buffer O: 10 m*M* Tris-HCl, pH 7.8
Buffer P: 10 m*M* Potassium phosphate, pH 6.5
Buffer Q: 50 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$–100 m*M* NaCl–0.25 m*M* ATP–10% (v/v) glycerol
Buffer R: 100 m*M* Potassium phosphate (pH 6.5)–10 m*M* 2-mercaptoethanol–1 m*M* EDTA–20% (v/v) glycerol
Buffer S: 10 m*M* Tris-HCl (pH 7.4)–1 m*M* 2-mercaptoethanol–1 m*M* EDTA–20 m*M* NaCl–20% (v/v) glycerol
Buffer T: 20 m*M* Tris-HCl (pH 7.8)–5 m*M* $MgCl_2$–1 m*M* dithiothreitol–0.1 m*M* EDTA–50 m*M* NaCl–20% (v/v) glycerol
Buffer U: 20 m*M* Potassium phosphate (pH 6.8)–1 m*M* 2-mercaptoethanol–1 m*M* EDTA–50 m*M* NaCl–20% (v/v) glycerol

Assays

Proteolytic activity is measured by following the degradation of [^{14}C]-methyl(apo)hemoglobin, [^{3}H]methyl-α-casein, [^{125}I]insulin to products soluble in 10% trichloroacetic acid (TCA). To prepare these substrates, crystalline beef hemoglobin (Sigma) is methylated with [^{14}C]formaldehyde (40–60 mCi/mmol, New England Nuclear) and bovine α-casein with [^{3}H]formaldehyde (85 mCi/mmol, New England Nuclear) according to the procedure of Rice and Means.[56] To increase its proteolytic susceptibility, heme is extracted from the hemoglobin by use of methylethylketone.[57] The resulting specific activity of ^{14}C-globin is about (2–4) $\times$ 10^6 cpm/mg, whereas that of ^{3}H-casein is (2–3) $\times$ 10^7 cpm/mg. [^{125}I]Insulin (New England Nuclear) is stored in the presence of 5% bovine serum albumin at −30°C.

Unless otherwise indicated, all the assays are carried out in buffer C in the presence and absence of 3 m*M* ATP. When assayed with globin or casein as substrates, each incubation mixture contains an appropriate amount of enzyme and 4–5 μg of ^{14}C-globin or 10–15 μg of ^{3}H-casein in a final volume of 0.5 ml. Assays using insulin as a substrate contain 5–15 μg of insulin (Eli Lilly Co., Indianapolis, Indiana), a trace amount of [^{125}I]-insulin (about 12,000–15,000 cpm), and the enzyme in a final volume of 0.5 ml. The incubations are performed for 1 hr at 37°C. Each reaction is stopped by the addition of 60 μl of 100% TCA and 40 μl of bovine serum albumin (30 mg/ml) as a carrier. The assay tubes are kept on ice for 30 min, and after centrifugation, 0.4 ml of acid-soluble products from [^{14}C]-globin or [^{3}H]casein hydrolysis are counted in 4 ml of Liquiscint (National Diagnostics, Parsippany, New Jersey). The products of [^{125}I]insulin hydrolysis are counted in an auto-gamma spectrometer.

In experiments with inhibitors the agents are incubated for 20–30 min at 37°C with the enzyme and buffer before the addition of substrate.

Preparation of Cell-Free Extract

Seventy-five grams (wet weight) of freshly grown, or frozen *E. coli* K12 cells (Grain Processing Co., Muscatine, Iowa) are suspended in 150 ml of buffer A at 4°C. The cell suspension is disrupted in a French press at 14,000 psi, the resulting homogenate is centrifuged at 31,000 *g* for 30 min, and the supernatant at 130,000 *g* for 3 hr. Following extensive dialysis against buffer B, the insoluble material is removed by centrifugation. This amount of cells yields 3–3.5 g of protein in the soluble extract. All the proteases to be described have been prepared from such extracts. How-

[56] R. H. Rice and G. E. Means, *J. Biol. Chem.* **246**, 831 (1971).
[57] F. W. J. Teale, *Biochim. Biophys. Acta* **35**, 543 (1959).

ever, protease La (the *lon* gene product) has been purified to near homogeneity by an alternative procedure[37] (see following discussion).

Fractionation of Cell-Free Extract on DEAE-Cellulose

The dialyzed cell-free extract (3 g protein) is applied to a DEAE-cellulose column (2.5 × 27 cm) equilibrated with buffer B. The column is washed with the same buffer until the A_{280} of the eluate is less than 0.05. The adsorbed proteins are then eluted with 1500 ml of a linear salt gradient (0–0.2 *M* NaCl). The flow rate is 100 ml/hr, and 15-ml fractions are collected. Alternate fractions are assayed for proteolytic activity against [^{3}H]-casein and [^{125}I]insulin in the presence and absence of 3 m*M* ATP. As shown in Figs. 1 and 2, five different peaks of proteolytic activity against these substrates are found. For convenience, they are designated peaks I–V, according to their order of elution from the column.[7] Although all the casein-hydrolyzing peaks also hydrolyzed globin, the activity against casein is much higher. The activity in peak IV, but in no other peak, is stimulated dramatically (4- to 20-fold) by ATP.

To obtain sufficient material for further purification, peaks from several DEAE-cellulose columns can be pooled. The following steps are described for enzyme preparations that would correspond to starting with 250–300 g of frozen cells.

Proteases in Peak I—Do and Re

Purification of Protease Do

Peak I (i.e., the fractions that did not adsorb to DEAE-cellulose) show strong hydrolytic activity against globin and casein. To resolve this activity further, fractions under peak I are pooled and concentrated by precipitating the proteins with solid ammonium sulfate added to 95% saturation. After centrifugation at 31,000 *g* for 30 min, the pellet is dissolved in buffer E and dialyzed against the same buffer. After removing the precipitated material by centrifugation, the proteins are loaded on a CM-cellulose column (1.5 × 12 cm) equilibrated with buffer E. The adsorbed proteins are eluted with a 0–0.3 *M* linear NaCl gradient. The flow rate is 35 ml/hr and 4-ml fractions are collected. Fractions are assayed in 50 m*M* Tris-HCl, pH 7.8. As shown in Fig. 3, the cation exchanger resolves the activity into two distinct peaks, referred to as proteases Do and Re. Both of these peaks hydrolyze globin and casein.

The active fractions corresponding to protease Do from the CM-cellulose column are pooled, concentrated by ultrafiltration through a

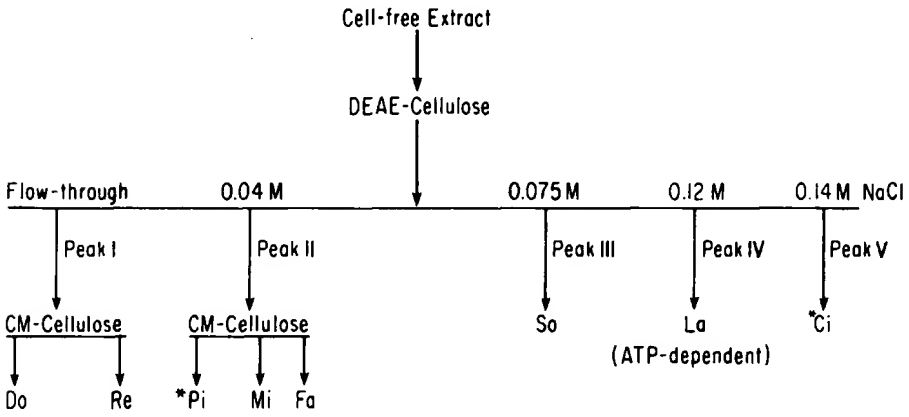

FIG. 1. Flowchart for the separation of *E. coli* proteases. *, Insulin-degrading proteases. (All others degrade globin and casein, but not insulin.)

PM-10 membrane, and dialyzed against buffer D. The dialyzed enzyme preparation is loaded on a Sepharose-6B column (2.0 × 100 cm) equilibrated with the same buffer. Fractions of 2 ml are collected at a flow rate of 20 ml/hr. Fractions containing protease Do activity are clearly separated from the nonenzymatic protein peak. The active fractions are pooled, concentrated by ultrafiltration, and stored at −30°C.

Properties of Do

Protease Do is located primarily in the cytoplasm.[39a] The enzyme has similar activity over a broad pH range from 6 to 8.5. The enzyme has an unusually large molecular weight of about 520,000, as determined by gel filtration on Sepharose-6B. Based on sucrose-density gradient centrifugation, the enzyme has a sedimentation coefficient of 16 S. In certain preparations the enzyme elutes with an apparent molecular weight of 320,000–350,000.[57a] The reason for this variability is unknown. The enzyme is inhibited by DFP (80% at 1 m*M*), and therefore it is a serine protease. It is not sensitive to metal chelators or sulfhydryl inhibitors.

Purification of Protease Re

Fractions exhibiting protease Re activity (Fig. 2) are combined, and precipitated by adding solid ammonium sulfate to 55% saturation. After centrifugation at 31,000 *g* for 30 min, the pellet is discarded, and the supernatant is brought to 80% saturation with ammonium sulfate. After

[57a] K. H. S. Swamy, C. H. Chung, and A. L. Goldberg, in preparation (1981).

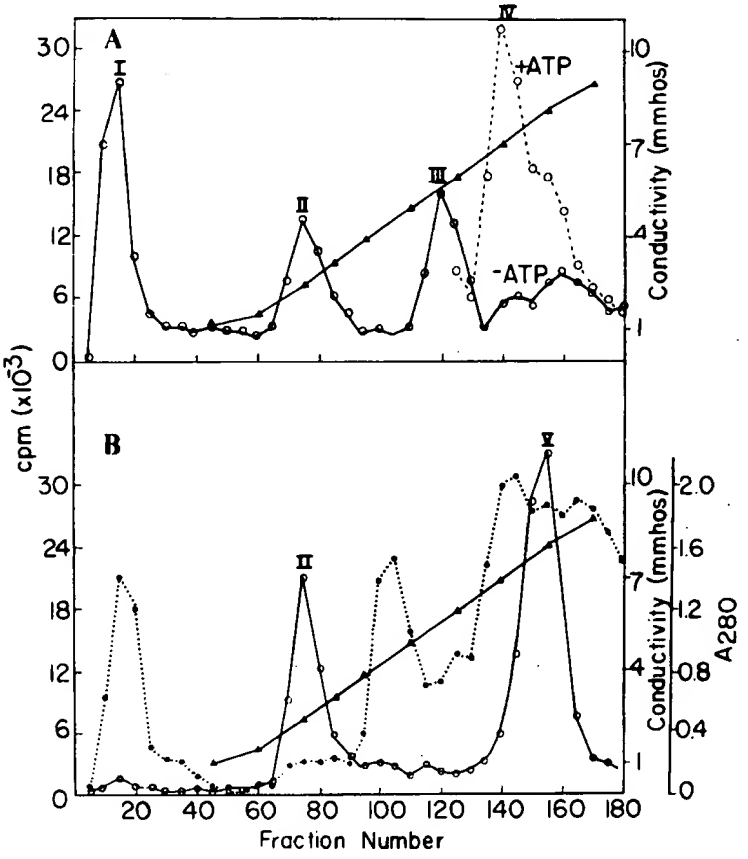

FIG. 2. DEAE-cellulose chromatography of proteolytic enzymes from *E. coli* K12. (A) Degradation of ^{3}H-casein measured in the absence (O—O) and presence (O---O) of ATP. ▲—▲, Conductivity. (B) Degradation of ^{125}I-insulin (O—O) is shown and compared with A_{280} (●· · ·●) and the conductivity (▲—▲). The dialyzed cell-free extract is adsorbed to a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0–0.2 *M* NaCl). The flow rate is 100 ml/hr and 15-ml fractions are collected. Alternate fractions are assayed for proteolytic activity against ^{3}H-casein and ^{125}I-insulin in the absence and presence of 3 m*M* ATP.

stirring the suspension for 2 hr at 4°C, the suspension is centrifuged at 31,000 *g* for 30 min; the pellet is then resuspended in buffer J and dialyzed against the same buffer. The enzyme preparation is applied to an Ultrogel ACA 44 (LKB) column (1.5 × 95 cm) equilibrated with buffer J. 2-ml fractions are collected at a flow rate of 15 ml/hr. The active fractions from the Ultrogel column are then pooled, dialyzed against buffer F, and loaded

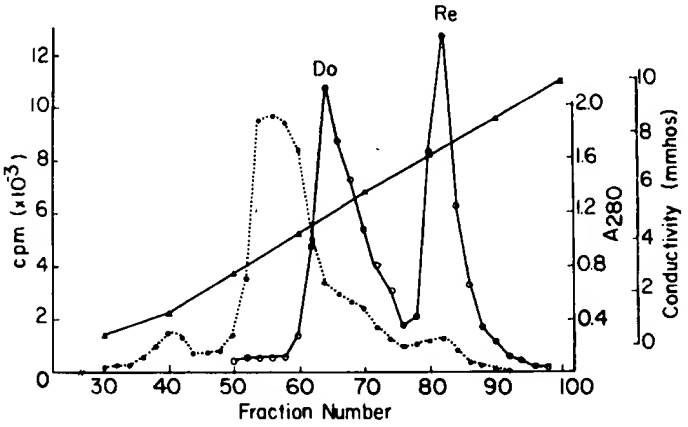

FIG. 3. CM-cellulose chromatography of proteases in peak I. The pooled peak I fractions from DEAE-cellulose are concentrated, dialyzed against buffer E, and applied to a CM-cellulose column (1.5 × 12 cm) equilibrated in buffer E. The absorbed proteins are eluted with 400 ml of 0–0.3 *M* linear NaCl gradient. The flow rate is 35 ml/hr and 4-ml fractions are collected. Hydrolysis of ^{3}H-casein (O—O); A_{280} (●· · ·●); conductivity (▲—▲).

on a DEAE–Sepharose column (0.5 × 9.5 cm) equilibrated with the same buffer. A linear gradient of 0 to 0.2 *M* NaCl is run at a flow rate of 20 ml/hr, and 1-ml fractions are collected. The peak of enzyme activity appears at about 75 m*M* NaCl.

Properties of Re

In intact cells protease Re is almost equally distributed between periplasm and cytoplasm.[39a] The enzyme has MW ~ 82,000 as determined by gel filtration on Sephadex G-75. Analysis of the enzyme on SDS–polyacrylamide gel electrophoresis shows a single band of MW 82,000. Thus the enzyme is composed of a single polypeptide chain.[57b]

The enzyme is active over the pH range of 7 to 8.5, with an optimal activity at pH 8.0. The enzyme is sensitive to inhibition by DFP (60% at 10 m*M*) and phenylmethylsulfonyl fluoride (PMSF) (60% at 10 m*M*). Therefore, it appears to be a serine protease, but it is also inhibited 70% by either EDTA (1 m*M*) or *o*-phenanthroline (1 m*M*). The enzyme is completely inhibited by L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) (1 m*M*), but not by 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK).

[57b] C. H. Chung, K. H. S. Swamy, and A. L. Goldberg, in preparation (1981).

Proteases in Peak II—Pi, Mi, and Fa

Purification

The fractions in peak II from the DEAE-cellulose column contain both casein- and insulin-degrading activities. They are pooled and concentrated by precipitating the protein with solid ammonium sulfate added to 95% saturation. After centrifugation at 31,000 *g* for 30 min, the pellet is dissolved in buffer D and dialyzed against the same buffer. The dialyzed enzyme preparation is loaded onto a Bio-Gel A-0.5m column (2.5 × 110 cm) equilibrated with buffer D. The flow rate is 25 ml/hr, and 3-ml fractions are collected. Alternate fractions are assayed against ^{3}H-labeled casein and [^{125}I]insulin. Proteolytic activities against both substrates appear in a single peak. The active fractions are pooled, dialyzed against buffer H, and applied to a CM-cellulose column (1.5 × 16 cm) equilibrated with the same buffer. After washing the column with 100 ml of buffer, the proteins are eluted with a 0 to 0.3 *M* linear NaCl gradient (350 ml total volume). Flow rate is 35 ml/hr, and 3-ml fractions are collected. Three peaks of proteolytic activity are resolved in this way (Fig. 4), but they can also be separated nicely with isoelectric focusing.[7] One hydrolyzes insulin, but not casein or globin, whereas the other two degrade these larger proteins but not insulin.

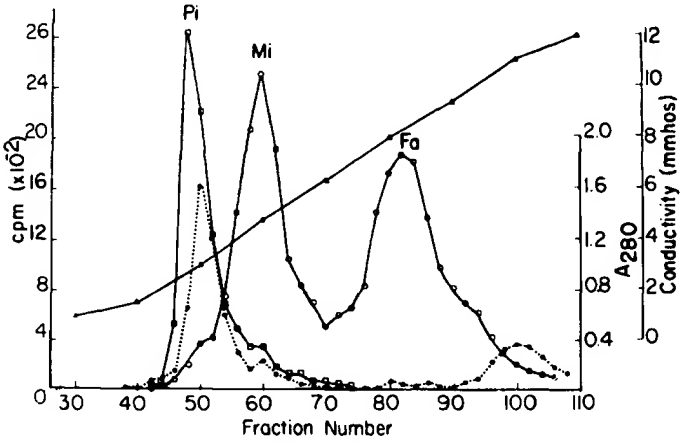

FIG. 4. CM-cellulose chromatography of proteases in peak II. The pooled Ultrogel AcA 34 fractions are dialyzed against buffer H and applied to a CM-cellulose column (1.5 × 16 cm) equilibrated in buffer H. The adsorbed proteins are eluted with 350 ml of 0–0.3 *M* linear NaCl gradient. The flow rate is 35 ml/hr and 3-ml fractions are collected. Hydrolysis of ^{125}I-insulin (□—□) and ^{3}H-casein (○—○); A_{280} (●···●); conductivity (▲—▲).

The insulin-degrading activity has been named protease Pi (periplasmic insulin-degrading enzyme) because of its localization exclusively in the periplasmic compartment.[39a] It is identical to protease III, described by Cheng and Zipser,[4] as discussed below. The two casein-degrading enzymes are named Mi and Fa. The active fractions corresponding to protease Mi and Fa are pooled, concentrated by ultrafiltration through PM-10 membrane, dialyzed against buffer C, and stored at −30°C.

The protease Pi (protease III) is further purified by chromatography on hydroxyapatite. The active fractions under the peak of protease Pi are pooled, dialyzed against buffer I, and applied to a hydroxyapatite column (1.5 × 14 cm) equilibrated with the same buffer. The enzyme is eluted with a linear gradient of 20–200 m*M* potassium phosphate, pH 7.4 (250 ml total volume). The flow rate is 45 ml/hr, and 2.5-ml fractions are collected. The fractions with highest activity are pooled, concentrated by ultrafiltration through PM-10 membrane, dialyzed against buffer C, and stored at −30°C. Cheng and Zipser have purified this enzyme to homogeneity by an alternative approach that involves many additional steps.[4]

Properties of Protease Pi

This enzyme is localized in the periplasm of the cell.[39a] It hydrolyzes insulin, glucagon, and the auto-α-fragment of β-galactosidase.[26] The optimum pH for the enzyme activity is 7.5. Protease Pi has MW ~ 110,000 as determined by gel filtration on Sephadex G-200. The enzyme activity is very sensitive to inhibition by EDTA (80% at 1 m*M*) and *o*-phenanthroline (50% at 1 m*M*). The enzyme activity is also inhibited by dithiothreitol by 60% at 5 m*M* but not by 2-mercaptoethanol. The enzyme is not inhibited by sulfhydryl inhibitors or inhibitors of serine proteases.

As shown by its physical properties and inhibitor sensitivity, protease Pi appears identical to protease III purified by Cheng and Zipser[4] using the auto-α-polypeptide as a substrate. In addition, the protease III-deficient mutant[29] lacks the insulin-degrading activity in peak II.[39a,58] Using oxidized insulin B chain as a substrate, Cheng and Zipser[4] have shown that protease III initially cleaves the peptide bond between tyrosine and leucine (16-17) and also cleaves between phenylalanine and tyrosine (25-26) at a slower rate.

Properties of Protease Mi and Fa

These two enzymes have different locations in the cell: protease Mi in the periplasm, protease Fa in the cytoplasm.[39a] Both enzymes degrade casein and globin. Protease Fa also degrades the auto-α-polypeptide.[58]

[58] C. H. Chung, K. H. Sreedhara Swamy, and A. L. Goldberg, unpublished observations.

Both these enzymes have MW ~ 110,000, as determined by gel filtration on Sephadex G-200.[58a]

Both Mi and Fa are serine proteases, although Fa is inhibited more strongly than Mi by DFP. At 10 m*M* concentration, DFP inhibits the casein-degrading activity of Mi by 55% and almost completely the activity of Fa. TPCK inhibits protease Fa (80% at 0.5 m*M*) but not Mi. Both are insensitive to inhibition by TLCK.

The activities of Mi and Fa are also sensitive to inhibition by metal chelators. EDTA inhibits protease Mi and Fa by 80% at 1 m*M*. *o*-Phenanthroline also inhibits Mi (63% at 1 m*M*) and Fa (80% at 1 m*M*). Sulfhydryl group-blocking agents do not affect either activity. Dithiothreitol inhibits the activity of Mi (35% at 1 m*M*) and Fa (67% at 1 m*M*), but 2-mercaptoethanol has no effect.

Protease in Peak III—So

Purification of Protease So

The activity against casein in peak III from the DEAE–cellulose column is pooled, and solid ammonium sulfate is slowly added to 45% saturation with stirring at 4°C. After additional stirring for 1 hr, the suspension is centrifuged at 31,000 *g* for 30 min, and the pellet is discarded. Ammonium sulfate is added again to bring the supernatant to 75% saturation, and the mixture is then stirred at 4°C for 2 hr. The pellet is collected by centrifugation at 31,000 *g* for 30 min, dissolved in buffer J, and then dialyzed overnight against the same buffer. The dialyzed material is applied to an Ultrogel AcA 34 column (2.5 × 120 cm) equilibrated with buffer J. Proteins are eluted with buffer J at a flow rate of 20 ml/hr, and fractions of 3 ml are collected. The fractions containing protease activity are pooled and dialyzed against buffer K at 4°C overnight. Insoluble proteins are removed by centrifugation at 31,000 *g* for 30 min. The supernatant is loaded on a CM-cellulose column (1.5 × 12 cm) equilibrated with buffer K. Proteins are eluted with a linear gradient of 0 to 0.3 *M* NaCl in buffer K. Fractions of 3 ml are collected at a flow rate of 30 ml/hr. Fractions with high protease activity are pooled, concentrated, and dialyzed against buffer L at 4°C for 8 hr.

The dialyzed protease preparation from the previous step is then applied to a DEAE–Sepharose column (1.5 × 5.6 cm) equilibrated with buffer L. Proteins are eluted with buffer L at a flow rate of 12 ml/hr, and fractions of 3 ml are collected. Protease So activity is eluted in the flow-through fractions, whereas all of the nonenzymatic proteins remain bound

[58a] K. H. S. Swamy and A. L. Goldberg, in preparation (1981).

TABLE I
PURIFICATION OF PROTEASE So

Step	Protein (mg)	Specific activity (units/mg protein)[a]	Purification factor (-fold)	Total units[a]	Yield (%)
DEAE-cellulose Peak III	369.8	0.5	1	184.9	100
Precipitates at 45–75% $(NH_4)_2SO_4$	214.1	1.2	2.4	256.9	140
Ultrogel AcA 34	61.0	4.7	9.4	286.7	155
pH precipitation	57.4	5.7	11.4	327.2	177
CM-cellulose	5.4	101.1	202.2	545.9	295
DEAE-Sepharose	0.16	1008.5	2017.0	161.4	87

[a] One unit is defined as 1 μg casein hydrolyzed per hour per milligram of protein.

to the resin. The active fractions are pooled and concentrated. These methods yield protease So with about a 2000-fold purification over that in peak III from DEAE-cellulose (Table I).

Properties of So

Protease So is a cytoplasmic enzyme of MW 140,000 as determined by gel filtration of Sephadex G-200.[39a,58b] On SDS–polyacrylamide gel electrophoresis, the enzyme shows a single band of MW 70,000. The native enzyme thus is a dimer of identical subunits. The purified enzyme appears homogeneous as determined by polyacrylamide-gel electrophoresis under denaturing and nondenaturing conditions.[58b] It is active over a pH range of 6–8, with maximal activity at 6.5. Because the enzyme is inactivated by DFP (60% at 10 m*M*) and PMSF (80% at 10 m*M*), it seems to be a serine protease. It is also inhibited by about 80% by pentamidine (1 m*M*). It is not sensitive to metal chelating agents or sulfhydryl inhibitors. The enzyme activity is completely inhibited by 1 m*M* TPCK but not by TLCK.

Protease in Peak IV—La: The ATP-Dependent Protease

Purification of Protease La

Two alternative approaches for the purification of protease La have been developed. (I) The first utilizes peak IV obtained from the DEAE-cellulose column between 0.105 and 0.15 *M* NaCl. This region shows

[58b] C. H. Chung and A. L. Goldberg, in preparation (1981).

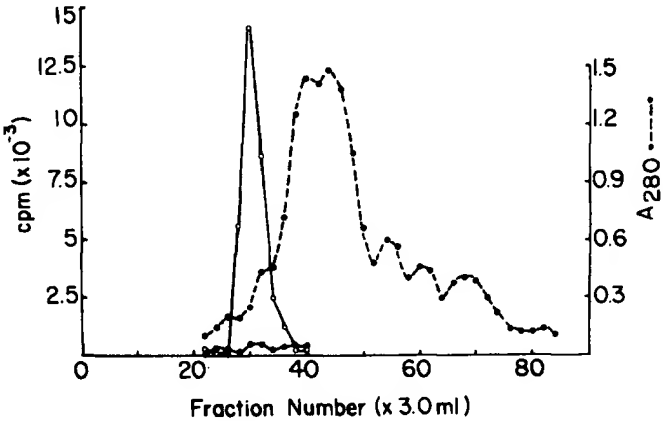

FIG. 5. Bio-Gel P-300 column chromatography of protease La. The pooled activity from the DEAE-Sepharose column is dialyzed against buffer Q, concentrated, and loaded to a Bio-Gel P-300 column (2.6 × 45 cm) equilibrated in buffer Q. Proteins are eluted with the same buffer. Fractions of 3 ml are collected at a flow rate of 10 ml/hr. Fractions are assayed for ^{3}H-casein hydrolysis in the presence (O—O) and absence (●—●) of 3 m*M* ATP. A_{280} (●-----●).

appreciable casein-degrading activity in the presence of 3 m*M* ATP but not in its absence (Fig. 2). The fractions with high ATP-dependent activity are pooled, dialyzed against buffer M, and loaded onto a DEAE-Sepharose column (1.5 × 30 cm) equilibrated with buffer M. The proteins are eluted with a 0–0.2 *M* NaCl gradient in buffer M, and a symmetrical peak of ATP-dependent activity came off the column at about 0.12 *M* NaCl concentration. Fractions with the highest activity are pooled, concentrated, and loaded onto a Bio-Gel P-300 column (2.6 × 45 cm) equilibrated with buffer Q. By this method, most of the nonenzymatic proteins can be well separated from the ATP-dependent protease activity (Fig. 5). For this purpose, we find Sephacryl S-300 is also useful. The active fractions are pooled and stored at −30°C. This approach yields a highly purified activity, which, however, is not homogeneous.

(II) As just discussed, protease La has recently been shown to be identical to the product of the *lon* (*CapR*) gene.[37b] Before this identification was achieved, Zehnbauer *et al.*[37] developed a procedure for isolating the polypeptide encoded by the *lon* gene, which on SDS–polyacrylamide gel electrophoresis appears to have MW 94,000. This protein shows an affinity for both DNA and ATP. Subsequent work[37a,37b] has shown that this purified material has ATP-dependent proteolytic activity. Purification of this enzyme has benefited from the use of *E. coli* in which the *lon* gene has

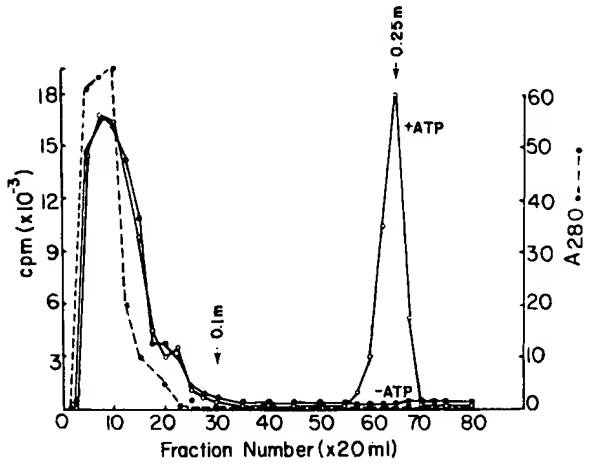

FIG. 6. Phosphocellulose column chromatography of protease La as described by Zehnbauer *et al.*[37] for the purification of *lon* gene product. Cell-free extract in buffer R is loaded onto a phosphocellulose column (2.6 × 20 cm) equilibrated with buffer R. The adsorbed proteins are eluted with 0.1–0.4 *M* linear phosphate gradient. Fractions are assayed for ^{3}H-casein hydrolysis in the presence (○—○) and absence (●—●) of 3 m*M* ATP. A_{280} (●---●) and conductivity (× · · · ×) are also shown. Data were kindly provided by Dr. L. A. Yeh.

been cloned on plasmid RGC 121/pJMC 40. Similar approaches, however, also work with wild-type bacteria.[58c]

Cell-free extracts are prepared by suspending 50 g of freshly grown *E. coli* K12 containing plasmid RGC 121/pJMC 40 (a strain containing the wild-type allele *lon*$^+$ both on the chromosome and on the plasmids) in buffer R. The cell suspension is disrupted in a French press at 14,000 psi and the resulting homogenate is centrifuged at 48,000 *g* for 30 min. Proteins at 20 to 25 mg/ml in buffer R is then applied to a phosphocellulose (Whatman P11) column (2.6 × 20 cm) equilibrated with buffer R. The adsorbed proteins are eluted with 0.1 to 0.4 *M* linear phosphate gradient. The ATP-dependent proteolytic activity is eluted at 0.25 *M* phosphate (Fig. 6). Appropriate fractions from the phosphocellulose column are dialyzed against buffer S and applied to a DEAE-cellulose column (2.6 × 9.5 cm) equilibrated with buffer S. The column is eluted in a stepwise fashion with buffer S containing 0.075, 0.15, 0.2, 0.25, and 0.3 *M* NaCl in 5-ml fractions at a flow rate of 15 to 20 ml/hr. Fractions (0.2 *M* NaCl) containing the ATP-dependent protease activity from the DEAE-cellulose column are dialyzed against buffer T and applied to an ATP-

[58c] L. A. Yeh and L. Waxman, personal communication.

agarose (agarose-hexane-adenosine 5'-triphosphate, Type 4, PL Biochemicals) column (0.5 × 2.5 cm) equilibrated with buffer T. Fractions are eluted in buffer T containing 5 m*M* ATP. The fractions containing the highest enzymatic activity are pooled, dialyzed against buffer U, and applied to a DNA–cellulose (prepared according to Litman[58d]) column (0.5 × 1.5 cm) equilibrated with buffer U containing 0.3 mg bovine serum albumin per milliliter. Proteins are eluted in a stepwise fashion with buffer U containing 0.5, 0.1, 0.2, 0.3, and 0.5 *M* NaCl. A convenient alternative to ATP- and DNA-affinity chromatographies is gel filtration on a Sephacryl S-300, as described above.[58e] The purified ATP-dependent protease is obtained in the 0.05 *M* NaCl fractions and are stored at −30°C.

Properties of La

The casein- or globin-degrading activity of protease La is completely dependent on the presence of ATP and Mg^{2+} ions. Other nucleoside triphosphates (CTP, UTP, or deoxyadenosine triphosphate) can replace ATP but are less effective than ATP. Mn^{2+} and Ca^{2+} can also replace Mg^{2+}. Nonmetabolizable analogs of ATP (5'-adenylyl-β,γ-imidodiphosphate and 5'-adenylyl-β,γ-methylene diphosphate) do not support proteolytic activity, although they do bind to the enzyme, apparently at the same rate as ATP.

Protease La (*lon* protein) has MW 450,000 as determined by glycerol-density gradient centrifugation or by gel filtration on Sephacryl S-300.[37b] On SDS–polyacrylamide gel electrophoresis, the purified *lon* protein shows a single band of MW 94,000.[37,37a] Thus the native enzyme appears to be a tetramer of identical subunits. It is present in the cytoplasm of the cell,[39a] but a similar (perhaps an identical) enzyme appears to be bound to the surface membrane in intact cells.[8] Because it is sensitive to DFP, it is a serine protease. The inhibition by *N*-ethylmaleimide and iodoacetamide indicates that sulfhydryl groups are important for activity. The proteolytic activity is also sensitive to inhibition by vanadate, quercetin, and Diol-9, potent inhibitors of ATPases.[59] Vanadate appears to form a transition-state analog with the enzyme, as is seen with various ATPases. Therefore, ATP cleavage seems essential for the proteolytic activity. In support of this conclusion, the purified enzyme has ATPase activity that is sensitive to vanadate and that is activated by protein substrate.[59a]

Clear stoichiometry between ATP hydrolysis and protein cleavage, however, has not been demonstrated. Protease La does not show protein kinase activity and does not catalyze adenylation of itself or of casein.[39]

[58d] R. M. Litman, *J. Biol. Chem.* **243**, 6222 (1968).

[58e] C. H. Chung and A. L. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.* in press (1981).

[59] F. S. Larimore, L. Waxman, and A. L. Goldberg, submitted for publication (1981).

[59a] L. Waxman and A. L. Goldberg, in preparation (1981).

Protease La in crude preparations and in the absence of glycerol (10–20%) is quite labile, and this property retarded attempts to purify it completely. The presence of organic solvents (above 1%), Triton X-100 above 0.1%, and temperatures above 37°C cause significant losses in enzyme activity. On storage at 4°C, the enzyme activity decreases about 30–40% per day. In the presence of 10 m*M* 2-mercaptoethanol or 3 m*M* ATP, these losses are reduced to less than 10%/day.[59] In addition, 3 m*M* ATP prevents the inactivation of the enzyme when incubated at 42°C for 1 hr. The stabilizing effects of ATP are further evidence that ATP binds directly to the protease in regulating its activity.[59]

Protease La differs in several important respects from the *recA* gene product, which cleaves certain regulatory proteins in an ATP-dependent reaction[5,17,35] (see preceding discussion). Adenosine 5′-*O*-(3-thiotriphosphate), a slowly metabolized analog of ATP, stimulates cleavage on the lambda repressor by *recA* proteins severalfold more effectively than ATP,[35] whereas it increases casein degradation by protease La only 10–20% as well as ATP. Thus proteolysis by *recA* does not seem to require ATP hydrolysis, although it contains an ATPase function. Mutants lacking a functional *recA* gene still contain protease La,[9] and thus these two ATP-dependent enzymes appear to be completely distinct proteins serving different physiological functions.

These properties differentiate this enzyme from any known proteolytic enzyme. In mammalian cells, as in *E. coli*, protein degradation requires metabolic energy,[1] and soluble extracts have been prepared from rabbit reticulocytes that degrade proteins in an ATP-dependent fashion.[60–62] An alkaline endoproteolytic activity that is stimulated 2- to 3-fold by ATP has been purified from such preparations.[63,64] It has similar molecular weight, and very similar proteases have been isolated from rat liver[65] and other tissues.[6] The ATP-dependent protease in *E. coli* differs from these enzymes in many properties.[59] For example, the mammalian enzymes are stimulated by nonmetabolizable analogs of ATP and by pyrophosphate and are not affected by vanadate.[6,63,64] Herskho, Rose, and co-workers have reported that ATP-dependent proteolysis in reticulocyte extracts[66–69]

[60] J. D. Etlinger and A. L. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 54 (1977).

[61] A. L. Goldberg, J. D. Kowit, J. D. Etlinger, and Y. Klemes, *in* "Protein Turnover and Lysosome Function" (H. Segal and D. Doyle, eds.), p. 171. Academic Press, New York, 1978.

[62] J. D. Etlinger and A. L. Goldberg, *J. Biol. Chem.* **255**, 4563 (1980).

[63] F. S. Boches and A. L. Goldberg, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **39**, 1682 (1980).

[64] F. S. Boches, L. Waxman, and A. L. Goldberg, in preparation (1981).

[65] G. N. DeMartino and A. L. Goldberg, *J. Biol. Chem.* **254**, 3712 (1979).

[66] A. Ciechanover, H. Heller, S. Elias, A. L. Haas, and A. Hershko, *Proc. Natl. Acad. Sci. U.S.A* **77**, 1365 (1980).

involves multiple components that can be resolved by chromatography on DEAE-cellulose. They suggested that ATP does not affect a protease but instead is required for the ligation of a small peptide (identified as ubiquitin) to the protein substrates. This alteration is supposed to enhance proteolytic susceptibility. The properties of the *E. coli* protease La are not consistent with such models. The bacterial enzyme does not require ubiquitin, and its activity is not stimulated by this polypeptide (which is found in *E. coli*).

Instead, ATP cleavage appears to influence the protease directly either by an allosteric activation or by some novel involvement in the hydrolytic mechanism. Furthermore, interaction of protease La with substrates somehow promotes ATP hydrolysis.[59a] One other important property of this enzyme is that DNA stimulates both the proteolytic and ATPase activities.[58e]

Protease in Peak V—Ci

Purification of Protease Ci

The insulin-degrading activity of peak V from the DEAE-cellulose column is pooled and dialyzed overnight against buffer E at 4°C. Insoluble proteins are removed by centrifugation at 31,000 *g* for 30 min. The supernatant fraction is adjusted to pH 7.8 by adding 200 m*M* Tris-HCl (pH 8.0), and solid ammonium sulfate is then added to 40% saturation. After stirring for 2 hr at 4°C, the suspension is centrifuged at 31,000 *g* for 30 min, and the pellet discarded. The supernatant is brought to 65% saturation with ammonium sulfate and stirred for 2 hr at 4°C. The pellet is collected by centrifugation at 31,000 *g* for 30 min, dissolved in buffer N, and dialyzed against the same buffer.

The dialyzed material is applied to an Ultrogel AcA 34 column (2.5 × 110 cm) equilibrated with buffer N. Proteins are eluted with the same buffer, and 3-ml fractions are collected at a flow rate of 20 ml/hr. The active fractions from the Ultrogel column are pooled, dialyzed against buffer O overnight at 4°C, and applied to a butyl-agarose column (1.0 × 18 cm) equilibrated with the same buffer. Protein is eluted by a linear gradient of 0 to 0.2 *M* NaCl in buffer O, and 3-ml fractions are collected at a flow rate of 30 ml/hr. The peak of enzyme activity appears at a NaCl concentration of about 80 m*M*.

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[67] H. Hershko, A. Ciechanover, H. Heller, A. L. Haas, and I. A. Rose, *Proc. Natl. Acad. Sci. U.S.A.* 77, 1783 (1980).

[68] A. Ciechanover, S. Elias, H. Heller, S. Ferber, and A. Hershko, *J. Biol. Chem.* 255, 7525 (1980).

[69] K. D. Wilkinson, M. K. Urban, and A. L. Haas, *J. Biol. Chem.* 255, 7529 (1980).

TABLE II
PURIFICATION OF PROTEASE Ci

Step	Protein (mg)	Specific activity (units/mg protein)[a]	Purification factor (-fold)	Total units[a]	Yield (%)
DEAE-cellulose					
Peak V	157.3	0.5	1	991.0	100
pH precipitation	111.1	9.25	1.5	1027.7	104
Precipitates at 45–65% $(NH_4)_2SO_4$	80.8	12.3	2.0	993.4	100
Ultrogel AcA 34	35.4	23.6	3.7	835.4	84
Butyl-agarose	2.6	141.6	22.5	368.2	37
Hydroxyapatite	0.6	352.5	56.0	211.5	21

[a] One unit is defined as 1 μg insulin hydrolyzed per hour per milligram of protein.

TABLE III
PROTEASES FROM *E. coli*

Proteases	Stimulation by ATP	Inhibitors	Molecular weight
Globin and casein-degrading enzymes			
Do	–	DFP	520,000
Re	–	DFP, EDTA, *o*-phenanthroline, TPCK	82,000
Mi	–	DFP, EDTA, *o*-phenanthroline	110,000
Fa	–	DFP, EDTA, *o*-phenanthroline, TPCK	110,000
So	–	DFP, TPCK	140,000
La	+	DFP, *N*-ethylmaleimide, EDTA	450,000
Insulin-degrading enzymes			
Pi (periplasmic)	–	EDTA, *o*-phenanthroline	110,000
Ci (cytoplasmic)	–	*o*-Phenanthroline, *p*-hydroxymercuribenzoate	125,000

The fractions containing insulin-degrading activity from the butyl-agarose column are pooled, dialyzed against buffer P at 4°C overnight, and loaded on a hydroxyapatite column (1.0 × 8.5 cm) equilibrated with the same buffer. After washing the column with the same buffer, proteins are eluted with a linear gradient of 10–200 m*M* potassium phosphate, pH 6.5. 2-ml fractions are collected at a flow rate of 20 ml/hr. The active fractions from the column are pooled, dialyzed against buffer O, concentrated by ultrafiltration, and stored at −30°C. The summary of purification is shown in Table II.

Properties of Ci

This enzyme has MW 125,000 as determined by gel filtration on Sephadex G-200.[70] It is located in the cytoplasm of the cell[39a] and has a sharp pH optimum at 7.5. Protease Ci is inhibited by 1 m*M* *o*-phenanthroline by about 80%, but not by EDTA or EGTA. The enzyme is also completely inhibited by *p*-hydroxymercuribenzoate (1 m*M*), but is not very sensitive to other sulfhydryl inhibitors. The antibiotics bacitracin (5 μg/ml) and globomycin (5–20 μg/ml) inhibit protease activity by 40% and 70%, respectively. This activity is stimulated 2- to 4-fold by Mn^{2+} (10 m*M*) and Co^{2+} (1 m*M*); Mg^{2+} and Ca^{2+} also stimulate, but less effectively. This protease hydrolyzes insulin, glucagon, and the amino-terminal fragments of β-galactosidase (auto-α). The distinct features of Ci and other *E. coli* proteases are summarized in Table III.

Acknowledgments

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[70] C. H. Chung, K. H. S. Swamy, and A. L. Goldberg, in preparation (1981).

[51] Nonlysosomal Insulin-Degrading Proteinases in Mammalian Cells

By RICHARD J. KIRSCHNER and ALFRED L. GOLDBERG

The characterization of intracellular proteinases is essential for a complete understanding of the regulation and specificity of intracellular proteolysis. For studying such proteinases, insulin and glucagon are inexpen-

ISBN 0-12-181980-9

[11] General Purification Schemes for Restriction Endonucleases[1]

By VINCENZO PIRROTTA and THOMAS A. BICKLE

The range of organisms used for the production of restriction enzymes and the various properties of these enzymes is such that it is difficult to devise a purification scheme of general application. The combination of polyethyleneimine precipitation and chromatography on heparin-agarose discussed in this article has a sufficiently wide applicability and a number of advantages to recommend it as the backbone of a general purification scheme or as a first approach in the isolation of new restriction endonuclease activities. In individual cases, however, additions or modifications of this procedure are necessary to optimize the results. In this article we will first present the basic procedure and then discuss alternatives or modifications suitable to certain particular cases.

Growth and Storage of Cells

The requirements and conditions of growth vary of course with the wide variety of organisms which produce restriction enzymes. In most cases the amount of enzyme per cell does not seem to vary substantially during the growth cycle, and it is therefore possible to allow the cells to grow to stationary phase before harvesting. This results in substantial saving in media and means that the cultures do not have to be monitored carefully. Most enzymes are stable in cell pastes stored at −70° for long periods of time, so that it is feasible to grow more cells than are actually needed for a preparation.

Opening Cells

Many different methods have been used to break open cells for restriction enzyme preparations. These include osmotic shock of lysozyme-prepared spheroplasts, explosive decompression in the French press, grinding with glass beads or alumina, and sonication. This last method is probably the most generally satisfactory and universally applicable so long as the quantity of cells to be opened does not exceed 20–300 g (wet weight). The general procedure most often used in our laboratory is described below.

[1] This work was supported in part by grants from the Swiss National Foundation for Scientific Research.

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Method. Thaw and suspend a frozen cell paste in an equal volume of buffer containing 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 7 m*M* 2-mercaptoethanol. Sonicate at maximum power of the sonicator until most of the cells are broken, taking care that the temperature does not rise above 10°. In practice this is best done by performing the sonication with the sample immersed in an ice-water bath and sonicating for periods of 30–60 sec allowing 2–3 min between bursts for the sample to cool down. It is very often difficult to decide when the sample has been sufficiently sonicated. For some bacteria the suspension will clear as the cells break, but this is far from being a general rule. The most generally reliable method of monitoring cell breakage is by observation in the light microscope. In general, a total sonication time of 5–10 min is sufficient.

Removal of Cell Debris and Ribosomes

Once the cells have been opened, it is necessary to remove unbroken cells and cell debris. Ribosomes may constitute as much as 30% of the total protein in a crude lysate. Their removal represents a considerable purification and improves the resolution of subsequent fractionations. This is achieved by centrifuging the crude lysate at 100,000 *g* for 1 hr. The presence of Mg^{2+} facilitates the pelleting of ribosomes which otherwise tend to form a loose layer which is easily disturbed by decanting. Fine cell debris which often packs poorly even after ultracentrifugation can sometimes be made to aggregate into larger, more firmly packing material by a single cycle of freezing and thawing of the cell extract. For large scale preparations, the high speed centrifugation is facilitated by a previous low speed centrifugation to remove the bulk of the coarse cell debris.

Removal of Nucleic Acids

It is essential to remove as much of the nucleic acids as possible from the cell extracts before chromatography on ion exchangers, particularly phosphocellulose, since the presence of nucleic acids changes the chromatographic properties of many enzymes, probably because the enzymes prefer to bind to the nucleic acid rather than to the ion exchanger.

Several methods have been used for this: gel filtration chromatography, fractional precipitation with ammonium sulfate, and specific precipitation with agents that precipitate mainly nucleic acids but not proteins. The first of these methods, gel filtration chromatography at high ionic strength has been widely used in restriction enzyme purifications, particularly by Roberts.[2] It suffers from the disadvantage that it is only

[2] R. J. Roberts, *Crit. Rev. Biochem.* **4**, 123 (1976).

feasible with fairly small scale preparations; the amount of column material needed for a large scale preparation becomes prohibitive, and the volume of the eluate is large and contains a high concentration of salt which must be removed by dialysis before subsequent purification steps. The advantages of the method are that it is fairly rapid and that the same column can be regenerated and used many times.

The most efficient way of removing nucleic acids is by precipitation. Traditionally, this has been done with streptomycin sulfate and more recently with polyethyleneimine (PEI).[3-5]

Method. A 10% (v/v) solution of PEI (Serva, practical grade) in water is prepared, and the pH is adjusted to 7.5 with HCl. The solution has a turbid appearance. This solution is added slowly and with stirring in the cold to the high speed supernatant to a final PEI concentration of 1%. After stirring for at least 30 min (and sometimes for as long as 16 hr) the precipitated nucleic acids are removed by low speed centrifugation. The ionic conditions are important for the success of the PEI precipitation. In low salt conditions (the buffer used for sonicating the cells) some proteins including many restriction enzymes, coprecipitate with the nucleic acids. In some cases the enzyme can be recovered from the PEI pellet by extraction with buffers containing 0.1–0.2 *M* NaCl. When this is possible the enzymes are often pure enough to use for some purposes without further purification[4,5] (see below discussion or criteria for purity). Other enzymes cannot be isolated in an active form from the PEI pellet. For these, the PEI precipitation must be performed in relatively high (0.1–0.2 *M*) concentrations of salt. Under these conditions the nucleic acids still precipitate but most of the proteins remain in solution. At present, we routinely do the PEI step at 0.2 *M* NaCl even for those enzymes that can be recovered from the pellet when the precipitation is done in low salt. This is because we have found that the recovery of some enzymes from the PEI pellet is variable, even though the degree of purification is high.

Polyethyleneimine itself would also interfere with chromatography. It can be removed, and the proteins concentrated, by precipitation with ammonium sulfate. Solid ammonium sulfate is added to the PEI supernatant slowly and with stirring to a final concentration of 70% of saturation. After all of the ammonium sulfate has dissolved, stirring is continued for a further 30 min, and the precipitated proteins are harvested by low speed centrifugation.

[3] A. H. A. Bingham, A. F. Sharman, and T. Atkinson, *FEBS Lett.* **76**, 250 (1977).
[4] J. Sümegi, D. Breedveld, P. Hossenlopp, and P. Chambon, *Biochem. Biophys. Res. Commun.* **76**, 78 (1977).
[5] T. A. Bickle, V. Pirrotta, and R. Imber, *Nucleic Acids Res.* **4**, 2561 (1977).

Chromatographic Procedures

For most restriction enzymes further purification will be obtained by one or more steps of column chromatography. The most popular, and until recently generally useful, column material is phosphocellulose, which has the advantages of selectivity for nucleic acid binding proteins and high capacity. It suffers from the disadvantages that it is chemically unstable and difficult to equilibrate. Recently, we have used heparin covalently linked to agarose as an alternative to phosphocellulose. Proteins which bind to nucleic acids apparently recognize heparin as a nucleic acid analogue and bind to the heparin-agarose column because of this affinity rather than by ion exchange. Heparin-agarose has the advantages of very high capacity, high selectivity for nucleic acid binding proteins, chemical stability, and ease of equilibration. Restriction enzymes usually bind to heparin more tightly than nonspecific nucleases and consequently eluted at high concentrations. Because of the very high capacity, it is possible to use small bed volumes, which means that the enzymes usually elute as sharp peaks in relatively small volumes. A procedure for the preparation of heparin agarose follows:

Preparation of Heparin-Agarose. Five hundred milliliters of settled bed volume of Biogel A 1.5 *M* or Sepharose 2B are washed twice with distilled water and suspended in 1.5 liters of distilled water. The suspension is placed on a magnetic stirrer in an ice bath, and a thermometer and a pH electrode are immersed in it. Twenty-five grams of CNBr are dissolved in 50 ml of cold dimethyl formamide, and the solution is added with stirring to the suspension of agarose. The pH is then adjusted to 10.5–11.5 with 5 *N* NaOH. As the reaction proceeds, HBr is produced and the pH drops. The pH should be maintained within the limits given above by the addition of 5 *N* NaOH until the rate of change of pH with time becomes negligible (30–60 min). The temperature should be maintained below 15° by the occasional addition of crushed ice. The suspension is collected on a fritted glass filter and washed with 5 liters of distilled water. All of the above operations should be carried out in a well-ventilated hood since CNBr is volatile and extremely toxic. The CNBr-activated agarose is suspended in 1 liter of 0.1 *M* $NaHCO_3$ containing 1 g of heparin. We have used Sigma grade 1 heparin (Catalogue No. H-3125), other grades may also be satisfactory. The suspension is stirred at 4° overnight, 50 ml of triethanolamine is added, and the suspension is stirred for a further 4 hr. The agarose is collected on a fritted glass filter and washed with 2 liters of 1 *M* NaCl and 5 liters of distilled water. The agarose is then suspended in an equal volume of the buffer that will be used for chromatography, in our case, 20 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA. Heparin-agarose made in this way and stored at 4° is stable for at least 2 years.

Use of Heparin-Agarose. Heparin agarose is used in a very similar way to ion exchange chromatography media with the only difference being that lengthy equilibration with starting chromatography buffers is unnecessary. The required volume of heparin-agarose is made into a 30% (v/v) slurry with the storage buffer supplemented with 7 m*M* 2-mercaptoethanol, degassed under vacuum and packed into the column. We normally use a bed volume of 20–25 ml per 100 g wet weight of cells and a bed height 5–10 times the diameter of the column. The columns are operated at a flow rate of 0.5–1 bed volumes per hour although higher flow rates have been used without apparent loss of binding capacity. They are washed with at least 2 bed volumes of chromatography buffer (20 m*M* Tris-HCl, 7 m*M* 2-mercaptoethanol, 0.5 m*M* EDTA). The sample, dialyzed against the chromatography buffer, is pumped onto the column which is then washed with a further 2 bed volumes of chromatography buffer. Restriction enzymes are eluted with a gradient of NaCl generally between 0 and 0.8 *M* NaCl made up in 10 bed volumes of chromatography buffer.

Other Column Materials. Depending on the enzyme and on the degree of purity required, it may be necessary to employ a second chromatographic step. Phosphocellulose and DEAE-cellulose are the most convenient available materials. Chromatography on phosphocellulose will not in general be a repeat of the heparin step. The two materials separate according to different principles, and the order of elution of two enzymes from the two different columns is frequently inverted. DEAE-cellulose has also been of use in some cases. The column can be equilibrated with the same buffer used for the heparin-agarose. No restriction enzyme so far examined binds to DEAE-cellulose at higher than 0.4 *M* salt. Recently, we have had good results with DEAE-Sephacel (Pharmacia) which has a cellulose matrix in spherical bead form. This material is supplied preswollen and equilibrated with Tris buffer and so needs very little preparation. In our hands, it gives sharper separations than the fibrous cellulose derivatives.

Other types of chromatography systems have also been employed for restriction enzyme purifications, including DNA cellulose or agarose[6] and hydrophobic interaction chromatography.[7] This last system might be particularly useful for those enzymes that have a tendency to precipitate in low salt, since the hydrophobic matrices are loaded at relatively high salt concentrations.

In cases where more than one restriction enzyme are present in the

[6] H. Schaller, C. Nüsslein, F. J. Bonohoeffer, C. Kurz, and I. Nietzschann, *Eur. J. Biochem.* 26, 474 (1972).

[7] R. E. Gelinas, P. A. Myers, G. H. Weiss, K. Murray, and R. J. Roberts, *J. Mol. Biol.* 114, 433 (1977).

same cell extract, heparin-agarose is often not a good choice as a first chromatographic step. Since heparin separates on the basis of affinity, two restriction enzymes are frequently poorly resolved. In the case of *Haemophilus aegyptius*, for example, the extract contains vast quantities of *Hae*III and moderate quantities of *Hae*II. On heparin-agarose these two enzymes elute very close to one another, and the *Hae*II activity is overshadowed by the *Hae*III which elutes slightly earlier. Instead the separation of the two activities is nearly complete on phosphocellulose where *Hae*II elutes first, followed by *Hae*III which is pure enough for sequencing use. If necessary, the *Hae*II fractions can be further purified, to remove traces of *Hae*III, with a small heparin-agarose column which has the additional effect of concentrating the enzyme. Another example is *Bgl*I–*Bgl*II. These two enzymes are frequently poorly resolved on heparin agarose or on phosphocellulose. In this case the best separation is obtained with DEAE-cellulose.

Problem Cases

In some instances the PEI precipitation is not sufficient to remove substances which interfere with subsequent steps. We have encountered this problem with *Mbo* and to a larger extent with *Mnl* and with *Taq*. In the case of *Mbo* and *Mnl*, the contaminating material which interfered with ammonium sulfate precipitation and with column chromatography could be removed together with the nucleic acids by precipitation with streptomycin at a final concentration of 5%. The supernatant was then subjected to ammonium sulfate precipitation before loading on heparin-agarose. In the case of *Taq*, we found that the extracts contained an acidic "slime" which interfered with binding of the enzyme to columns even after PEI precipitation. The slime could be removed by applying the 35–75% ammonium sulfate cut to a DEAE-cellulose column. A relatively large bed volume is necessary since the slime, as well as the enzymes, binds to the column. Although the column gives poor resolution, the active fractions now bind well to heparin-agarose affording good separation of *Taq*I from *Taq*II.

Assay Procedures

The most useful assay procedure for chromatographic separations of restriction enzymes is electrophoresis of digests of DNA on slab gels of polyacrylamide or agarose. This is described in detail elsewhere in this work (see Section V). Here, we would just like to emphasize that the conditions of incubation may be important for the interpretation of the

results. It is very easy to overdigest the DNA by incubating the samples too long with too much enzyme. This may have two results. First, a complete digest is obtained through a large part of the gradient making it difficult to decide where the enzyme peak is located. Second, if two different enzymes are separated by the gradient the more abundant may "swamp out" the activity that is present in lesser amounts.

Criteria for Purity

Enzymes may be purified to different degrees, depending on the use that will be made of them. For mapping or comparative studies, an enzyme need only be purified to the point where digests of DNA give sharp bands on agarose or polyacrylamide gels. For DNA sequencing work this is not enough; enzymes have to be completely free from contaminating 3'- or 5'-exonucleases, single-stranded-specific nucleases, phosphatases, etc. This is best tested directly with the aid of end-labeled substrates.

Relatively few attempts have been made to purify restriction enzymes to the point where they are suitable for enzymatic and protein chemical studies, that is, to homogeneity. This is probably because, until now, interest has centered on the application of the enzymes to nucleic acid studies rather than on the enzymes themselves.

Storage of Restriction Enzymes

Purified restriction enzymes are generally stored at $-20°$ in buffers containing 50% glycerol. The glycerol can be added directly to the enzyme-containing fractions from the last step of the purification, or the fractions can be dialyzed against buffer containing 50% glycerol. In this last case, a considerable concentration of the enzyme is obtained. Highly purified enzyme preparations can lose activity upon storage. This can often be prevented by adding autoclaved gelatin or bovine serum albumin to a final concentration of 50–100 μg/ml. Some enzymes, (*Eco*RI, *Pst*I, etc.) also require a neutral detergent, such as 0.2% Triton X-100, to stabilize them.